KUOPION YLIOPISTON JULKAISUJA D. LÄÄKETIEDE 410
KUOPIO UNIVERSITY PUBLICATIONS D. MEDICAL SCIENCES 410

DONALD WLODKOWIC

Selective Targeting of Apoptotic Pathways in Follicular Lymphoma Cells

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium ML1, Medistudia building, University of Kuopio, on Monday 18th June 2007, at 12 noon

Institute of Clinical Medicine
Department of Clinical Microbiology
University of Kuopio

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ISBN 978-951-27-0670-9
ISBN 978-951-27-0747-8 (PDF)
ISSN 1235-0303

Kopijyvä
Kuopio 2007
Finland

Wlodkowic, Donald. Selective targeting of apoptotic pathways in follicular lymphoma cells. Kuopio University Publications D. Medical Sciences 410. 2007. 97 p.
ISBN 978-951-27-0670-9
ISBN 978-951-27-0747-8 (PDF)
ISSN 1235-0303

ABSTRACT

The seminal discovery that the *BCL2* gene inhibits cell death rather than promotes cell proliferation gave the foundation for a nowadays widely appreciated theory that impaired tumor cell death is a decisive stage in a multi-step carcinogenesis. Recognition of the central role of mitochondria and Bcl-2 family members in the regulation and propagation of cell demise has, thus, recently uncovered novel targets for selective anti-cancer therapies. In this context, we have for the first time demonstrated monotherapeutic potential of a small molecule Bcl-2 antagonist, HA14-1, against follicular lymphoma (FL) cells with t(14;18) translocation and Bcl-2 overexpression. In agreement with others we have shown that cytotoxicity mediated by a small molecule Bcl-2 inhibitor HA14-1 in FL cells proceeds through rapid (4h) dissipation of the $\Delta\Psi_{m,}$ generation of ROS and caspase-dependent apoptosis. Moreover, we were first to prove the applicability of combinatorial treatment of HA14-1 and selected conventional chemotherapeutics (dexamethasone and doxorubicin) in FL cells. In the following work we investigated the quantitative relationship between $\Delta\Psi_m$ loss and caspase activation in HA14-1 treated cells. Employing both pharmacological inhibitor studies and novel state-of-the-art multiparametric flow cytometry assays, we revealed that following HA14-1 treatment caspase activation occurs solely as a consequence of mitochondrial breach. Additionally, we provided new evidence that HA14-1-evoked apoptosis appears to be at least partially PT-dependent. We also for the first time addressed the cell cycle specificity of HA14-1 action using multivariate flow cytometry approaches.

As the interest in the role of ER and Golgi during induction/execution of apoptosis has been gaining momentum, they simultaneously attract growing appreciation in the development of novel anti-cancer therapies. Pertinent to the therapy of B-cell malignancies we report here for the first time the effects of an ER-Golgi transport inhibitor, Brefeldin A (BFA), alone and in combination with a small molecule Bcl-2 inhibitor HA14-1 or death receptor trigger, in the human FL cell lines bearing t(14;18) translocation. Of importance for future anti-cancer regimens, small molecule Bcl-2 antagonist, HA14-1 and agonistic anti-Fas mAb significantly enhanced BFA- mediated cytotoxicity and apoptosis, revealing novel and previously unexplored avenues to enhance ER-stress mediated cell killing in B-cell malignancies.

Finally, as basic studies advance towards their ultimate translational goals there is a need for effective and rapid analytical methods allowing high-throughput detection of diverse cell demise modes. In this context patented SYTO® probes are gaining increasing interest as easy to use markers of apoptosis. Herein, applying the state-of-the-art multiparametric flow cytometry and multicolor cell imaging we identified for the first time different SYTO16 staining characteristics upon apoptotic and oncotic stimuli. We also tracked similarities and discrepancies between SYTO16 and $\Delta\Psi_{m,}$ sensitive probe, tertramethylrhodamine methyl ester (TMRM), demonstrating that stimulation with mitochondrial uncoupler FCCP and a small-molecule Bcl-2 inhibitor, HA14-1, induce distinct staining profiles with the decrease in TMRM fluorescence preceding the loss of SYTO16 fluorescence. To our knowledge this is the first report demonstrating such a distinct behavior of SYTO16 and TMRM and our data demonstrate that loss of SYTO16 is caspase-dependent, as is not a mere indicator of $\Delta\psi_m$ dissipation, postulated previously by some authors.

National Library of Medicine Classification: QU 375, QY 95, QZ 350, WH 525
Medical Subject Headings: Apoptosis; Brefeldin A; HA14-1; Cell Death; Cell Line, Tumor; Flow Cytometry; Genes, *BCL*-2; Lymphoma, Follicular; SYTO 16

ACKNOWLEDGEMENTS

Experiments for this study were conducted at the Department of Clinical Microbiology, University of Kuopio, during the years 2004-2007.

I would like to express my deepest gratitude for my principal investigator, Professor Jukka Pelkonen, for providing working space at the Department, obtaining founding sources and most of all for allowing me a profound exploration of both my scientific passions: translational cancer research and flow cytometry.

For the excellent technical assistance, every day lab-chats and friendship I would like to especially thank Eila Pelkonen. Thanks a lot for all the berries collected from wild Finnish forests where you have been frequently meeting bears and packs of wolfs.

My sincere thanks go also to: Pirkko-Liisa Kankkunen for the excellent administrative support in management of my fellowships and grants; Dr Tuomas Virtanen from the Department of Clinical Microbiology, University of Kuopio for relaxing chats about digital photography during short coffee-breaks in my intense work.

I would like to thank Arto Koistinen from the Bio-Mater Centre at University of Kuopio for training, substantial help and advice during transmission electron microscopy experiments.

Special regards go to my sincere friends Dr Magdalena Kacprzak and Dr Krzysztof Pluta from the Department of Molecular Cancer Biology, Biomedicum at the University of Helsinki. I cannot even describe your support and mental input during weekends and evenings spend together, especially in dark, wintry days in Kuopio. Krzysztof you are an unbeatable master of home-made beer. I miss a lot the time spent together both in Kuopio and later on in Turku.

Warm regards and credits for all chats and discussions I direct to all my colleagues, but especially to: Dr Artur Kostrzewa, from the Department of Biology and Environmental Sciences, Karol Marcinkowski University of Medical Sciences in Poznan; Dr Robert Kutner, from the Health Sciences Center, Louisiana State University in New Orleans; and Dr Rinako Nakagawa from the Division of Cancer Sciences and Molecular Pathology, University of Glasgow in Glasgow.

I am sending extremely warm hugs to my sincere friend and collaborator Professor Andrzej Deptala, head of the Department of Hematology, Oncology and Internal Medicine at the CSKMSWiA in Warsaw. Many thanks for all our fruitful discussions, your invaluable help and advice. I am very pleased that we are continuing further our flourishing collaboration.

Especially warm regards also go to my previous principal investigator and sincere friend Professor Krzysztof Wiktorowicz, head of the Department of Biology and Environmental Sciences at the Karol Marcinkowski University of Medical Sciences in Poznan. Thanks a lot for your help whenever I needed it and most of all the encouragement which propelled me to Kuopio.

I express my deepest appreciation for Dr Simon Brown from MRC Centre for Inflammation Research, University of Edinburgh in Scotland for our always refreshing, intense and stimulating discussions about clearance of apoptotic cells and molecular mechanisms of phagocytosis. It really gave me a completely innovative perception of the field.

I am sending my gratitude to Dr Alison Michie and Professor Tessa Holyoake from the Division of Cancer Sciences and Molecular Pathology, University of Glasgow for hosting me as a visiting research fellow in their lab at the end of 2006.

I would like to send an individual bear hug to my old comrade Jarek Jadrzyk. Thanks a lot for the time spend together during my short and seldom visits in a homeland and for you endless sense of humor helping my always during my ups and downs when being in Poznan.

The official reviewers of the thesis: Professors Zbigniew Darzynkiewicz (Brander Cancer Research Institute, NYMC, Valhalla, NY, USA) and Professor Piotr Smolewski (Department of Hematology, Medical University of Lodz, Copernicus Memorial Hospital, Lodz, Poland), are deeply accredited for their priceless feedback. I am incredibly grateful for all your helpful remarks and corrections provided to this thesis.

I contribute this dissertation to my beloved fiancée Joanna who worked with me side by side in the lab during the days and nights. Joanna only you know all the drawbacks I had encountered while working in Kuopio. I will remember forever our tremendous frustrations but also our fabulous excitements while working on our projects. I know that only thanks to your eternal love and encouragement I have survived and stayed on my track. Thanks a lot for your courage, for our crazy expeditions and adventures across Europe and for the time spend always together no matter what happens. I apologize for my hereditary stubbornness and appreciate your patience at work and at home.

Present work was financially supported by the University of Kuopio and the Kuopio University Hospital.

During my stay at the University of Kuopio I was awarded by personal fellowships from the Centre for International Mobility (CIMO) and the Finnish Cultural Foundation (Northern Savo branch).

Finally, I would like to forward my sincere apologies to the numerous authors whose admirable work could not be cited due to thesis length limitations.

Kuopio 05/04/2007

Donald Wlodkowic

ABBREVIATIONS

AIF	apoptosis inducing factor
ANT	adenine nucleotide transporter
AO	acridine orange
APC	allophycocyanine
AS	antisense
AVBB	Annexin V Binding Buffer
Bcl-2	B-cell lymphoma 2 protein
BFA	Brefeldin A
BH	Bcl-2 homology
CARD	caspase-activation recruitment domain
CI	combination index
CLL	chronic lymphocytic leukemia
CsA	cyclosporine A
CypD	cyclophilin D
Da	Dalton
Dex	dexamethasone
DHE	dihydroethidine
DISC	Death-Inducing Signaling Complex
DKO	double knockout
DMSO	dimethyl sulfoxide
Dox	doxorubicin
Endo G	endonuclease G
ER	endoplasmic reticulum
FACS	fluorescently activated cell sorting
FADD	Fas associated death domain
FBS	fetal bovine serum
FCS	fetal calf serum
FCM	flow cytometry
FITC	fluoroscein isothiocyanate
FL	follicular lymphoma
FLICA	fluorochrome-labeled inhibitors of caspases
fmk	fluoromethylketone
HA14-1	ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate
HtrA2	high-temperature requirement protein A2
IAP	inhibitor of apoptosis protein
IC_{50}	inhibitory concentration 50%
ICAD	inhibitor of caspase-activated deoxyribonuclease
IMM	inner mitochondrial membrane
IMS	mitochondrial intermembrane space
kDa	kilo Dalton
LC_{50}	lethal concentration 50%
LSC	laser scanning cytometry
mAb	monoclonal antibody
MAC	mitochondrial apoptosis-induced channel
MAGE-3	melanoma associated antigen 3
MEFs	mouse embryonic fibroblasts
MFI	mean fluorescence intensity

MLM	mitochondria localization motif
MOMP	mitochondrial outer membrane permeabilization
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
NAC	N-acetylcysteine
NAO	*nonyl*-acridine orange
OD	optical density
OMM	outer mitochondrial membrane
PARP	poly (ADP-ribose) polymerase
PCD	programmed cell death
P-gp	P-glycoprotein
PI	propidium iodide
PS	phosphatidylserine
PTP	permeability transition pore
ROS	reactive oxygen species
RT	room temperature
SDF-1	stromal cell-derived factor-1
Smac/DIABLO	second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI
tBid	truncated Bid
$tBclX_L$	truncated $BclX_L$
TMRM	tetramethylrhodamine methyl ester
UPR	unfolded protein response
VDAC	voltage-dependent anion channel
VEGF	vascular endothelial growth factor
Vin	vincristine
zVAD	benzyloxycarbonyl-Val-Ala-Asp

LIST OF ORIGINAL PUBLICATIONS

The PhD thesis is based on the following original publications:

I. Skommer J, **Wlodkowic D**, Mätto M, Eray M, Pelkonen J (2006) HA14-1, a small molecule Bcl-2 antagonist, induces apoptosis and modulates action of selected anticancer drugs in follicular lymphoma cells. Leukemia Res. 30(3):322-331.

II. **Wlodkowic D**, Skommer J, Pelkonen J (2006) Multiparametric analysis of HA14-1 triggered apoptosis in follicular lymphoma cells. Leukemia Res. 30(9):1187-1192

III. **Wlodkowic D**, Skommer J, Pelkonen J (2007) Towards an understanding of apoptosis detection by SYTO dyes. Cytometry A;71A(2):61-72

IV. **Wlodkowic D**, Skommer J, Pelkonen J (2007) Brefeldin A triggers apoptosis associated with mitochondrial breach and enhances HA14-1- and anti-Fas-mediated cell killing in follicular lymphoma cells. Leukemia Res (in press: doi:101016/j.leukres.2007.03.008)

The thesis includes also previously unpublished data.

Contents

1. Introduction

Every fifth person in the developed world will suffer from cancer in their lifespan. Therefore, the understanding of the molecular basis of cancer and mechanisms underlying tumor resistance to chemotherapy is of utmost importance for the development of novel and efficient treatment protocols.

Follicular lymphoma (FL), a malignancy of follicle center B cells, is the commonest type of indolent non-Hodgkin´s lymphoma (NHL) (Horning and Rosenberg, 1984; Horning, 1993). It is a heterogeneous and presently still incurable malignancy, characterized by a variable clinical course associated with frequent relapses and increasing chemoresistance to conventional anti-cancer regimens (Johnson et al, 1995). FL is generally characterized by the t(14;18) translocation that results in over-expression of anti-apoptotic Bcl-2 protein, a feature widely linked to the impairment of apoptosis and often correlated with enhanced resistance to conventional chemotherapy (Cleary et al, 1986; Vaux et al, 1988; Kirkin et al, 2004; Skommer et al, 2006). With the classical chemotherapy being frequently ineffective in achieving complete remission in FL patients, the quest for more selective, apoptosis targeted therapies is still ongoing (Green and Kroemer, 2005; Armstrong, 2006; Linder and Shoshan, 2006).

Classical chemotherapy regimens often trigger a mitochondrial (intrinsic) pathway of apoptosis, featured by the release of cytochrome c and/or other proteins residing in the mitochondrial intermembrane space leading to cell demise (Kim, 2005; Kim et al, 2006). This pathway is tightly regulated by the complex regulatory network of anti-apoptotic, and pro-apoptotic (multidomain and BH3-only) Bcl-2 family members. The ultimate efficacy of the conventional anticancer therapy can, thus, be heavily influenced by altering the fine balance between the members of the Bcl-2 family (Spierings et al, 2005; Skommer et al, 2007). Recognition of mitochondria and Bcl-2 family members as the master regulators of cell demise has recently uncovered novel targets for anti-cancer therapies, and indeed encouraging response has been initially achieved in pre-clinical and early clinical trials (Gardner, 2004; Oltersdorf et al, 2005, Chauhan et al, 2006; Konopleva et al, 2006). Recent discovery of alternative cell death pathways, which involve function of until recently underappreciated organelles such as endoplasmic reticulum, Golgi apparatus and lysosomes, increases further the promises for selective anti-cancer therapy. Targeting diverse cell death pathways in

malignant cells slowly emerges as an alternative approach to override the commonly existing defects in apoptotic machinery (Ferri and Kroemer, 2001; Jäättela, 2004; Linder and Shoshan, 2006).

Finally, the rapid progress in the modern field of tumor cell death requires also effective and reliable methods allowing high-throughput assessment of a multitude of critical parameters in the studied cell population. Up to date only a flow cytometry (FCM) and laser scanning cytometry (LSC) deliver a single cell, high speed, multiparameter capability, permitting unparalleled correlation of different cellular events at a time (Darzynkiewicz et al, 1997; Bedner et al, 1999; Deptala et al, 2001; Huang et al 2005). In this regard multiparametric flow cytometry as well as laser scanning cytometry protocols can prove useful to study the temporal and quantitative relationship between a plethora of apoptotic attributes, important in basic and clinical cancer research studies (Darzynkiewicz et al, 1997; Halicka et al 1997; Smolewski et al 2003). Thus, development of novel functional probes and thorough understanding of the exact mechanisms underlying properties of existing ones are of utmost importance for the progress in cell necrobiology (Darzynkiewicz et al, 1997). In this context the recently described SYTO probes are slowly gaining interest as sensitive and easy to use markers of apoptotic cell death in basic and clinical applications (Frey, 1995; Poot et al 1997; van der Pol et al, 2003). However, the phenomenon underlying differential SYTO staining of apoptotic cells vis-à-vis normal cells is not fully elucidated as yet.

In the present study, applying a multitude of flow cytometry protocols, emerging apoptosis targeting avenues (including a small molecule Bcl-2 inhibitor; HA14-1 and an ER-Golgi vesicular transport disrupting drug; Brefeldin A) were explored in the recently established follicular lymphoma cell lines (Eray et al, 2003). Moreover, novel insights into mechanisms underlying discrimination between normal and apoptotic cells by SYTO probes were investigated using multiparameter flow cytometry and multicolor cell imaging platforms.

2. Literature overview

2.1 Tumor cell death

Cancer is one of the most common causes of death in Western societies. Current statistics indicate rising trends in cancer incidence in the developed world (Cancer Research UK database http://info.cancerresearchuk.org/cancerstats). Thus, the hope to finally eradicate cancer is the main motivation driving the extensive cancer research studies over last two decades and this trend will probably continue for the decades to come.

Initial clinical perspective was that cancer is mainly characterized by the accelerated, uncontrolled proliferation of transformed cells leading to the accumulation of malignant clones in diverse organs, their subsequent dysfunction and fatal outcome for the patient. Thus it is of no surprise that majority of research activities over the past decades were targeting the cell cycle and development of novel, cell cycle selective drugs is still ongoing (Halicka et al, 1997; Schwartz and Shah, 2005). The profound contribution of tumor cell death in the process of tumorigenesis has only recently been brought to attention (Leist and Jaattela, 2001; Evan and Vousden, 2001; Crighton and Ryan, 2004). Moreover, the intense advancement in basic cancer research allowed our current emerging understanding that cancer originates not only from accumulation of genetic lesions leading to deregulated cell proliferation and impaired cell death but also from profound changes in the unique tumor microenvironment influencing both of them (Kern et al, 2004; Haiat et al, 2006). It is the combination of malfunctions in all those three processes that favors tumor development, its metastasis and finally resistance to conventional chemo- or radio- therapy (Haiat et al, 2006; Meinhardt at al 1999).

Tumor cell demise as such is an important event in the elimination of abnormal malignant cells and thus delivers *in vivo* a strong means of tumor suppression (Crighton and Ryan, 2004). Any genetic abnormalities incapacitating cell death mechanisms deliver, thus, a strong advantage for cancerous cell to succeed in evading both the cell intrinsic, self-check procedures and external immune surveillance (Meng et al., 2006). The avoidance of programmed cell death in tumorigenesis has been recently appreciated as one of the “hallmarks of cancer” (Hanahan and Weinberg, 2000). Although the burden of data shows that the elimination of malignant cells

depends heavily on classical apoptotic pathways of cell demise, the evidence is mounting that other non-apoptotic mechanisms may effectively contribute to tumor suppression and even deliver novel anti-cancer targets (Jäättela 2004; Kroemer and Jäättela 2005; Levine and Yuan, 2005; Bröker et al, 2005; Kim, 2005; Kim et al, 2006). A plethora of studies indicate that initiation of classical apoptosis is a common outcome of successful anti-cancer therapy (Lowe et al, 1993; Kaufmann and Gores, 2000; Ghobrial et al., 2005). Thus a reliance of many anti-cancer drugs on apoptotic pathways to kill malignant cells is believed to be required for the successful cancer eradication and widespread dysfunctions in apoptotic machinery often render cancerous cells resistant to conventional chemotherapy (Zhivotovsky and Orrenius, 2003; Ghobrial et al., 2005; Kim, 2005). In this context, multitude of successful pre-clinical and early phase clinical studies illustrate that modulation of a diverse molecular signaling pathways responsible for tumor cell demise may be effectively utilized with a selective therapeutic intent (Ashkenazi, 2002; Meng et al, 2006; Letai, 2005; de Thonel and Eriksson 2005; Kaufmann and Steensma 2005; Linder and Shoshan, 2005; Wright and Duckett, 2005; Bouchier-Hayes et al, 2005; Lavrik et al 2005). There is a substantial hope that an increase in our understanding of the mechanisms responsible for tumor cell death will lead to the development of novel tumor-selective anti-cancer regimens (Zhivotovsky and Orrenius, 2003; Debatin, 2004; Green and Kroemer, 2005; Fehrenbacher and Jäättela, 2005; Fischer and Schulze-Osthoff, 2005; Ghobrial et al., 2005).

2.2 Many ways to cross the Hades gates

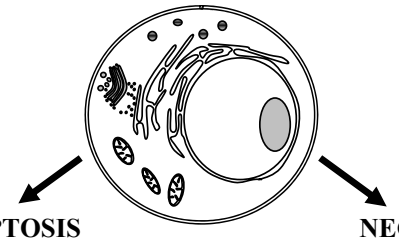
Extracellular and intracellular stresses reaching every normal cell in human body can initiate tumorigenic changes. Generally in an attempt to avoid transformation cell can either:

- enter cell cycle arrest and try to repair damage and then if successful reverse arrest and enter again cell cycle
- enter cell cycle arrest and if the threshold of damage exceeds the repair capacity survive but become senescent (discontinue its proliferative capacity – become a *"reproductively -dead"*)
- succumb to programmed cell death if the damage exceeds the repair capacity, if repair processes have been unsuccessful or if initiation of senescence program is impossible (Okada and Mak, 2004).

For many years it has been generally perceived that the cell can disassemble in two morphologically and biochemically distinct processes (Kerr et al, 1972; Lockshin and Zakeri, 2001). Classical view described apoptotic programmed cell death (PCD) and necrotic death being as a Dr Jekyll and Mr. Hyde for the cell commitment to death. In this view apoptosis was the tightly regulated execution whereas necrosis was nothing more then a passive and unregulated demise of the cell associated with ATP depletion and characterized by rapid rupture of the plasma membrane, release of cell constituents to the tissue environment that may lead to tissue inflammation and scar formation (Kerr et al, 1972; Darzynkiewicz et al 1997; Leist and Jäättela 2001). Based on our current knowledge, indeed, the cell that is undergoing classical apoptosis demonstrates multitude of characteristic and tightly regulated morphological and biochemical features (Kerr et al, 1972; Darzynkiewicz et al 1997; Danial and Korsmeyer, 2004). Some of the most characteristic attributes of apoptosis include: dehydration followed by cell shrinkage, loss of the mitochondrial membrane potential, activation of caspases and serine proteases, chromatin condensation, high molecular weight (HMW) followed by low molecular weight (LMW) DNA cleavage, nuclear fragmentation, alterations in the plasma membrane function leading to the exposure of the phosphatidylserine and increased plasma membrane permeability to small cationic probes, zeosis, disassembly of the cell into the apoptosis bodies and finally engulfment and elimination of the cell remnants by the professional phagocytes and/or the neighboring cells (Darzynkiewicz et al 1997; Petit et al 1997; Vermes et al, 2000; Danial and Korsmeyer, 2004; Ziegler and Groscurth, 2004; Gregory and Brown, 2005) (Fig 1). The final outcome of apoptotic cell demise avoids ultimately the uncontrolled release of cellular constituents to the surrounding environment and protects the tissue from the development of strong inflammatory response. Activation of transglutaminase that leads to extensive crosslinking of protein prevents their release in soluble form and also has anti-inflammatory consequences. For a long time apoptotic cell death has, thus, been perceived as a cell death program that may selectively eradicate cancer with minimal adverse effects to the host. Recently this view has been challenged by some authors (Brown and Vernon-Wilson, 2005). The evidence is slowly accumulating that excessive *in situ* presence of apoptotic cells may in fact be deleterious and deliver strong, unwanted immunomodulatory stimuli. Thus, the overwhelming apoptotic incidence exceeding the phagocytosis clearance capacity may initiate or exacerbate autoimmunological pathologies (Brown and Vernon-

Wilson, 2005). This remarkable view on apoptotic cell death with prospectively serious clinical implications is to be explored during coming years (Dr Simon Brown – personal communication).

Fig. 1 Hallmarks of apoptosis and necrosis



APOPTOSIS	**NECROSIS**
Morphological features	***Morphological features***
Cell dehydratation & shrinkage	Cell swelling
Loss of pseudopodia or microvilli	Nuclear and mitochondrial swelling
Detachment from the surface	Vacuolization of cytoplasm
"Blebbing" of plasma membrane	Rupture of plasma membrane
Chromatin condensation	Dissolution of chromatin
Nuclear fragmentation	Dissolution of cell constituents and attraction of inflammatory cells
Preservation of mitochondria	
Disassembly of the Golgi apparatus	
Formation of apoptotic bodies	
Shedding of apoptotic bodies	
Engulfment and elimination by phagocytes/neighboring cells	
Biochemical features	***Biochemical features***
Preservation of ATP levels	Mitochondrial dysfunction
Loss of mitochondrial membrane potential	Rapid depletion of intracellular ATP
Release of cytochrome c	Lack of caspase activation
Activation of caspases	Rapid loss of plasma membrane integrity
Activation of endonucleases	
DNA fragmentation	
Extensive phosphorylation of histone H2AX	
General preservation of plasma membrane integrity (increased permeability only for very small cations e.g. YO-PRO1)	
Externalization of phosphatidylserine on the outer leaflet of plasma membrane	
Activation of transglutaminase	

Recent reports have also provided further insights into the mechanisms of the programmed death sentence and led to the characterization of several alternative cell death modes (caspase independent PCD, autophagy, necrosis-like PCD, mitotic catastrophe) with serious connotations in cancer development and treatment (Leist and Jäättela, 2001; Jäättela, 2002; Lockshin and Zakeri, 2002; Okada and Mak, 2004; Edinger and Thompson, 2004; Abraham and Shaham, 2004; Hail et al, 2006) (Tab 1).

Based on the studies exploiting pharmacological inhibitors of caspases and genetic *knock-out* and *knock-down* approaches it has become evident that classical attributes of apoptosis may not always be manifested and the underlying mechanisms and ways of cell disintegration strongly depend on the cell type, cellular context (e.g. unique tumor microenvironment), nature of cellular insults (or initiator signals) and their intensity (Leist and Jäättela, 2001; Okada and Mak, 2004; Levine and Yuan, 2005; Lockshin and Zakeri, 2004; Edinger and Thompson, 2004; Hail et al, 2006). Recent observations have also delivered strong evidence of profound interorganelle cross-talk and involvement of diverse protease cascades leading to a finely orchestrated cellular disintegration. Classical apoptotic PCD as described by Kerr et al., in 1972 seems, thus, to no longer have exclusive rights to be called programmed.

Table 1 Current concepts on the complexity of cell demise modes (based on: Leist and Jäättela, 2001; Okada and Mak, 2004; Jäättela, 2004; Kroemer and Jäättela, 2005)

Cell demise mode	Distinctive morphological features	Distinctive biochemical features
Classical apoptosis	- Strong condensation of chromatin - Cell shrinkage - Preservation of cellular organelle - Cell membrane blebbing Formation of apoptotic bodies	- Absolute requirement of caspase cascade activation - Internucleosomal DNA fragmentation - Phosphatidylserine exposure
Caspase-independent apoptosis-like programmed cell death	- Chromatin condensation less pronounced than in classical apoptosis - Varying gradation and combination of apoptotic features possible	- Activation of caspases not necessary to execute the program although possible - Common activation of other proteases: cathepsins, calpains, serine proteases - DNA fragmentation less pronounced - Phosphatidylserine exposure often observed
Autophagy	- Partial chromatin condensation - Formation of double/multi layered autophagosome vacuoles dependent on ATG genes - Cell membrane blebbing possible	- Initially perceived as caspase independent although recent reports indicate possible cross-talk with classical apoptosis - Lack of DNA fragmentation - Increased lysosomal activity

Mitotic catastrophe	- Formation of giant polykaryons - Lack of chromatin condensation - Lack of cell membrane blebbing	- At initial stages caspase independent although final rerouting to caspase dependent execution is possible - Initiated by a violation of G2 checkpoint of the cell cycle and premature entry to mitosis
Necrosis-like programmed cell death (*classified also as aborted apoptosis*)	- Lack of geometric chromatin condensation or condensation forming loose speckles - Varying scale and combination of apoptotic features possible	- Initial caspase cascade activation possible with common subsequent inhibition and re-routing to alternative pathways - Predominantly random degradation of DNA - Phosphatidylserine exposure possible
Necrosis (*classified also as accidental cell death or cell lysis*)	- Lack of geometric chromatin condensation, dissolution of chromatin - Organelle and cell swelling - Lack of cell membrane blebbing - Rapid rupture of plasma membrane	- Lack of protease cascade activation - Random degradation of DNA (no DNA laddering) - Rapid and uncontrolled release of cell constituents
Senescence ***("cell zombie")***	- Appearance of characteristic heterochromatic foci - Characteristic flattened cytoplasm - Increased cellular granularity - Lack of cell membrane blebbing	- Caspase independent - Initiated by a shortening of telomeres and cell entry into irreversible cell cycle arrest (replicative senescence) - Profound changes in metabolism and activation of senescence-associated β-galactosidase (SA-β-gal)

Complicating is also fact that, depending on cellular/stimulus context, the same cell can often re-route its disintegrating pathways from caspase dependent cascades to alternative failsafe mechanisms (Bröker et al, 2005; Chipuk and Green, 2005). These influential discoveries initiated an ongoing debate aiming at the definition and classification of different modes of cell death which is of particular importance for the development of novel, selective anti-cancer agents (Darzynkiewicz et al, 1997; Zhivotovsky, 2004). The general term apoptosis, exploited commonly in many research articles, tend sometimes to misinterpret the actual mechanisms underlying cell suicide program (Leist and Jäättela, 2001; Zhivotovsky, 2004). Therefore it has

been recently postulated to restrict the term apoptosis to only the traditional cell demise program featuring all "hallmarks of apoptotic cell death", namely: activation of caspases as a absolute requirement to execute cell death; tight compaction of chromatin to geometric figures as a result of caspase dependent DNase (CAD) action; appearance of distinctive cellular morphology with preservation of organelle, cell shrinkage, plasma membrane blebbing and nuclear fragmentation followed by formation of apoptotic bodies (Leist and Jäättela, 2001; Zhivotovsky, 2004; Ziegler and Groscurth, 2004). The use of the general term apoptosis/apoptotic events should be rather substituted by the name of a particular morphological and/or biochemical parameters measured by the investigator. Some authors proposed to use the term apoptosis only to define the caspase-mediated cell death (Blagosklonny, 2000). It is also advisable to always exploit a plethora of different assays to cross-analyze action of e.g. novel anti-cancer compounds and bear in mind that the characteristic changes in cell morphology revealed by light or electrom microscopy still remains the gold standard in the ultimate classification of a cellular demise mode (Darzynkiewicz, 1997; King, 2000; Smolewski et al 2003). Proper experimental approaches will help to avoid any potential misclassifications as the evidences accumulate that the roads to cellular death represent a much more diverse and interconnected course than previously anticipated (Ferri and Kroemer, 2001; Leist and Jäättela, 2001; Okada and Mak, 2004; Chipuk and Green, 2005; Bröker et al, 2005).

2.3 Classical apoptosis program

Despite our advancements in the understanding a diversity of programmed cell deaths, the regulation, execution and importance of the classical apoptotic program in tumor surveillance and chemotherapeutic drug action is so far the best understood. Also, at least in the case of hematological malignancies the classical apoptotic program seems to predominate over the alternative back-up mechanisms in cell response to a range of intra- and/or extracellular stressors (Rassidakis et al, 2003; Brown and Attardi, 2005; Skommer et al, 2007). It is conceivable, however, that elaborate intracellular cross-talks between classical and alternative programs exist in every cell type and depending on the circumstances may participate in sensing, initiation and amplification of the ultimate cell dismantling (Ferri and Kroemer, 2001; Maag et al, 2003).

As described earlier one of the hallmarks of typical apoptosis is the activation of unique cysteine aspartyl-specific proteases having a conserved consensus site QACXG containing active cysteine, called caspases (from **c**ysteinyl **asp**artate-specific prote**ases**) (Thornberry and Lazebnik, 1998; Earnshaw et al, 1999; Zhivotovsky, 2003; Boyce et al, 2004; Lavrik et al, 2005; Ho and Hawkins, 2005). Similar absolute requirement for the cleavage after aspartate residue is sheared in mammals only by serine protease granzyme B, making caspases a truly unique group of proteases (Zhivotovsky, 2003; Fadeel and Orrenius, 2005). In mammals there are probably at least fourteen members of the caspase family which form a closely related family of proteases. Nevertheless at present only eight caspases are known to participate in execution of apoptotic cell dismantling (caspases 2, 3, 6, 7, 8, 9, 10, 12) whereas the rest of the caspase family participates in cytokine processing and inflammatory responses (caspases 1, 4, 5, 13) (Zhivotovsky, 2003; Boyce et al, 2004; Lavrik et al, 2005). Under normal physiological conditions caspases are constitutively expressed in the cytoplasm as zymogens (32 – 56 kDa) with very low intrinsic activity. They consist of: N-termini regulatory prodomain, a large subunit (17 – 21 kDa), linker region and a small subunit (10 – 13 kDa) (Earnshaw et al, 1999; Zhivotovsky, 2003). In response to initiator signal pro-caspases become proteolytically cleaved at specific aspartate residues between the large and small subunit and their pro-domains are being removed by the secondary proteolytical cleavage. Released small and large subunits dimerize and form a heterodimer which subsequently dimerizes again to form a fully active caspase heterotetramer (Earnshaw et al, 1999; Zhivotovsky, 2003; Boyce et al, 2004; Lavrik et al, 2005).

Caspases can be divided into two subclasses with regards to their function and the position in the apoptotic cascade:

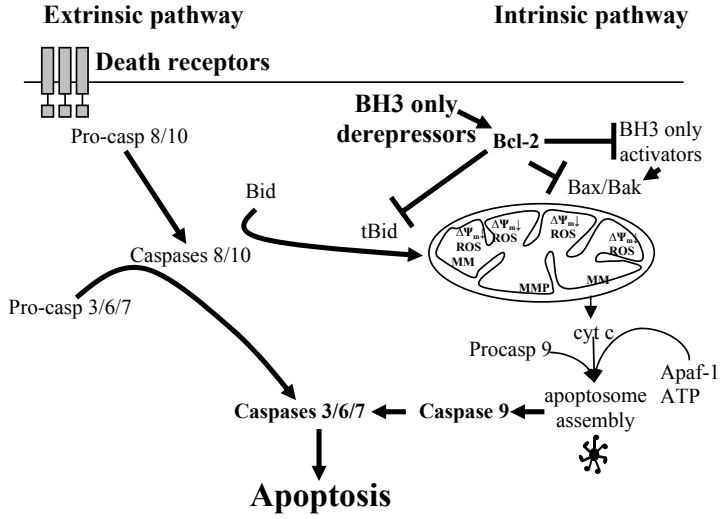
- initiator caspases required for the commencement of the proteolytic cascade (caspase 2, caspase 8, caspase 9, caspase 10 and caspase 12)
- executioner caspases being responsible for proteolytic cleavage of cell substrates leading to cell dismantling and appearance of the distinctive apoptotic morphology (caspase 3, caspase 6, caspase 7) (Zhivotovsky, 2003; Boyce et al, 2004; Lavrik et al, 2005)

Initiator caspases feature long, regulatory prodomains with characteristic protein interaction motifs. Regulatory prodomains are responsible for interaction with signal initiator complexes where close proximity allows self-proteolysis forced activation of

initiator caspases (Cain et al 2002; Cain, 2003; Danial and Korsmeyer, 2004). In this context initiator caspases 8 and 10 are characterized by a tandem DED motif (death effector domains) and caspases 2, 9, 12 are characterized by a single CARD motif (caspase-activation recruitment domain) at the N-termini of the molecule. Executioner caspases lack full-size regulatory prodomains and their activation depends on the action of upstream initiator caspases and/or alternative mechanisms leading to their proteolytic activation (Ferri and Kroemer, 2001; Bröker et al, 2005; Chwieralski et al, 2006). Once activated, caspases function in an orchestrated proteolytic cascade leading to its self-amplification and cleavage of specific cell substrates e.g.: ICAD, lamin A, actin, fodrin, gelsolin, poly-ADP-ribose polymerase (PARP) (Earnshaw et al, 1999; Zhivotovsky, 2003; Lavrik et al, 2005). Cleavage of cellular constituents does not obligatorily lead to their orchestrated disintegration but often to: modifications in target protein function, alterations in target protein activity level, uncovering alternative function of target protein and even activation of enzymatic function. The typical example is ICAD/CAD (**I**nhibitor of **C**aspase **A**ctivated **D**Nase/**C**aspase **A**ctivated **D**Nase) system where cleavage of the ICAD provides means to activate CAD, its translocation to nucleus and characteristic apoptotic DNA fragmentation.

Archetypically caspase activation can proceed though the two separate ways leading to proteolytic cascade activation: extrinsic and intrinsic pathways (Fig 2).

Fig. 2 Simplistic view of extrinsic and intrinsic pathways of apoptotic cell demise



Mounting evidence suggests, however, that caspase cascade can also be directly and efficiently launched from other intracellular organelle, like: nucleus, endoplasmic reticulum and Golgi apparatus with or without contribution of mitochondrial (intrinsic) pathway (Ferri and Kroemer, 2001; Maag et al, 2003; Hicks and Machamer, 2005). These fascinating aspects with direct connection to future anti-cancer therapies will be pursued further in the following paragraphs.

2.3.1 Extrinsic pathway

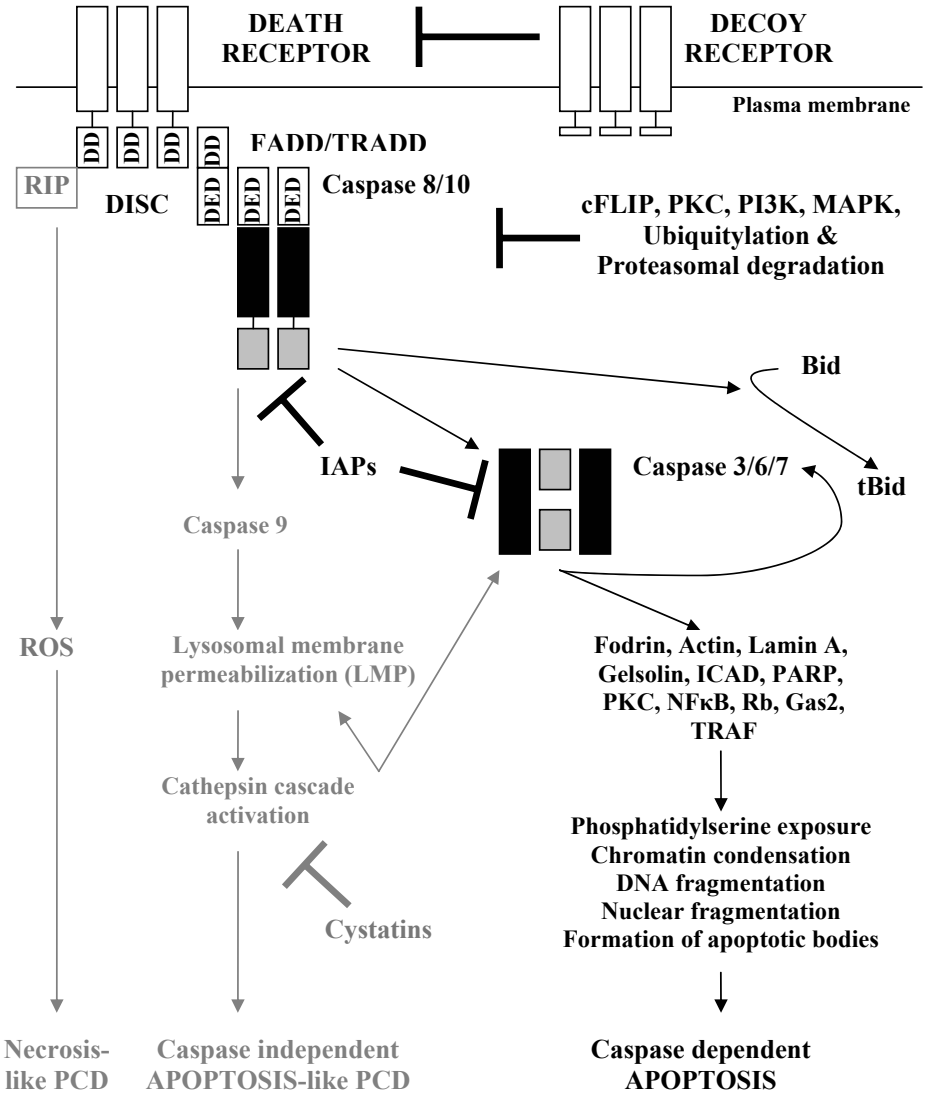
The most studied and thus far the best understood pathway of activating caspases is a receptor-linked pathway that necessitates ligation of the distinctive group of transmembrane receptors belonging to the Tumor Necrosis Factor α (TNF α) superfamily, commonly called death receptors (DR) (Fig. 2, 3) (Golstein, 1997; Ashkenazi and Dixit, 1999; Ashkenazi, 2002; Aggarwal, 2003; Thornburn, 2004; Thonel and Eriksson, 2005). Although some of DRs can transmit prosurvival signaling and at least under some circumstances promote cell growth, their ability to induce apoptosis is remarkable and probably corresponds to one of their main physiological functions (Ashkenazi, 2002; Aggarwal, 2003; di Pietro and Zauli, 2004; Park et al 2006). Up to date six distinctive DRs have been identified: TNFR1, CD95 (FasR), TRAIL R1 (DR4) and TRAIL R2 (DR5) (TNF-Related Apoptosis Inducing Ligand Receptors), DR3 and DR6 (Golstein, 1997; Ashkenazi and Dixit, 1999; Krammer, 2000; Ashkenazi, 2002; Bhardwaj and Aggarwal, 2003; Fulda and Debatin, 2004; Wajant et al, 2005). Important roles in regulation of immune system functions and tumorigenesis have been attribiuted to all death receptors. DRs are synthesized as monomeric transmembrane molecules whose extracellular N-termini contain three to four characteristic cysteine-rich domains (CRDs). The collective feature shared by all death receptors is a protein interaction motif located on the cytosolic tail of the molecule and called death domain (DDs) (Tartaglia et al, 1993; Ashkenazi, 2002). In addition to DRs, non-functional, so called decoy receptors (lacking or having truncated forms of DDs) have recently been described. They are being perceived as a modulatory mechanism for DRs signaling as decoys compete with DRs for the cognate ligands and appear not to have any supplementary biological functions (Sheridan et al 1997; Ashkenazi and Dixit, 1999; Ashkenazi, 2002; Kimberley and Screaton, 2004).

Death receptors' ligands belong to the TNF family cytokines and comprise of: TNF α (TNFR1 ligand), CD95L/Fas (Fas ligand), TRAIL (DR4/DR5 ligand) (Ashkenazi and Dixit, 1999; Ashkenazi, 2002; Aggarwal, 2003; Thorburn, 2004; Thonel and Eriksson, 2005). Little is still known about ligands for DR3 and DR6, and some reports point at TL1A and TWEAK as potential ligands for DR3. Also biological function of DR3 and DR6 remains up to date elusive. All ligands are synthesized as homotrimeric (type 2) transmembrane proteins possessing a highly conserved extracellular C-terminus (approximately 30% amino acid identity among superfamily) which can be proteolytically cleaved off to form a soluble ligand (Ashkenazi, 2002).

Activation of death receptors is executed by the ligation of the specific ligand (membrane bound or soluble) or agonistic monoclonal antibody which forces receptor trimerization, subsequent recruitment of specific adaptor proteins to their cytosolic death domains and formation of the DISC (**D**eath-**I**nducing **S**ignaling **C**omplex) (Fig. 3) (Ashkenazi, 2002; Thornburn, 2004; Thonel and Eriksson, 2005). Some reports postulate that death receptors can in fact exist in a pre-associated trimerized form and receptor ligation is rather responsible for conformational changes leading to the recruitment of cytosolic adaptor protein FADD (**F**as **A**ssociated **D**eath **D**omain) to the death domain (Siegel et al, 2000; Thornburn, 2004). Interestingly, there are indications that action of a number of anticancer drugs on the membrane lipid composition can lead to DRs clustering in cell membrane lipid rafts and their activation without involvement of specific ligands (Dimanche-Boitrel et al, 2005). Orthodox model assumes that during formation of the DISC, adaptor protein FADD (consisting of two protein interaction domains: DD – death domain and DED – death effector domain) binds directly or through secondary adaptor TRADD (**T**NF**R** **A**ssociated **D**eath **D**omain - required by TNFR1) to the receptor's death domain. Subsequent formation of DISC requires attraction of caspase 8/10 via homotypic interactions between N-terminally positioned DED motifs on the caspases and DED motif within FADD molecule (Fig. 3) (Chinnaiyan et al 1995; Ashkenazi, 2002; Thornburn, 2004; Thonel and Eriksson, 2005). Recruitment of pro-caspase 8/10 molecules allows then their homotypic dimerization, followed by proximity forced processing (self-proteolysis) and formation of active caspase 8/10 heterotetramers (Fig. 3) (Thornburn, 2004). Active caspases 8/10 subsequently initiate proteolytic cascade though activation of caspase 3/6/7 and cleavage of Bid molecule which

interconnects death receptor with mitochondrial (intrinsic) pathway of apoptosis (Fig. 2, 3).

Fig. 3 Extrinsic pathway of apoptosis with links to caspase-independent cell demise pathways (based on: Leist and Jäättela, 2001; Ashkenazi, 2002; Thorburn, 2004; Thonel and Eriksson, 2005; Gyrd-Hansen et al, 2006)



Recent discoveries cast, however, a shadow on the necessity of the self-proteolysis to achieve catalytic activity by caspase 8 (Thornburn, 2004). It appears that dimerization

step completely suffices for launching full activity of caspase 8 and processing step is neither essential nor sufficient for this event. It can probably only: (i) influence a stability of the formed dimers,; (ii) to some extend modulate its substrate specificity and/or, (iii) participate in the undocking of active caspase 8 from the DISC (Boatright et al, 2003; Donepudi et al, 2003; Thornburn, 2004; Dr Guy Salvensen – personal communication). Based on those data one should bear in mind that the data based on common assays exploiting detection of cleaved forms of caspase 8/10 on the Westernblots may no longer be adequate (Thornburn, 2004). Moreover, in line with the above data caspase 8 has been recently reported to be shed off inside the apoptotic vesicles resulting in heavily underestimated activity when assessed by flow cytometry approaches (Packard et al, 2001).

Fascinatingly, specificity of caspase 8 has lately also been challenged adding completely new dimension to our understanding of the death receptor pathway and further complicating the complexity of cell demise pathways. New light has been shed by Jäättela and colleagues (Gyrd-Hansen et al, 2006) on the direct caspase 8-mediated cleavage and activation of caspase 9 (Fig. 3). Namely, caspase 8-mediated processing of pro-caspase 9 after TNF ligation occurs at a different site than in apoptosome-dependent system (Asp349 instead of Asp353). Those breakthrough findings are bulldozing current dogma of sole caspase 9 activation after release of cytochrome c in the mitochondrial pathway of apoptosis (Fig 2). Remarkable evidence also exists linking the death receptor signaling with necrosis-like PCD, which appears to involve receptor interacting protein (RIP) and elaborate generation of reactive oxygen species (ROS) (Fig. 3) (Vercammen et al, 1998; Holler et al, 2000; Leist and Jäättela, 2001).

Regulation of death receptor pathway is achieved by the action of decoy receptors, inhibitor of apoptosis proteins (IAPs), FLICE inhibitory protein (cFLIP) and a plethora of signaling cascades involving PKC, PI3K and MAPK. Ubiquitinylation and proteasomal degradation of caspase 8 has recently been postulated to play a role in the regulation of extrinsic pathway (Ashkenazi, 2002; Thonel and Eriksson, 2005).

2.3.2 Intrinsic pathway

The second conventional pathway of caspase activation involves the participation of mitochondrion and is, as a result, termed the mitochondrial (intrinsic) pathway of apoptosis (Fig. 2) (Danial and Korsmeyer, 2004; Green and Kroemer, 2005). Over the past years, the understanding of the crucial role of mitochondria and their regulators

in the control of apoptosis began to emerge. Early evidence connecting mitochondria with apoptotic cell demise conceived that pro-apoptotic and pro-survival functions of these organelles are quite diverse. Our progressing knowledge indicates that, at least in vertebrates, this pathway of cell death is a universal response mechanism to a variety of physiological stresses, intended to eliminate superfluous and/or damaged cells (Green and Kroemer, 2005; Spierings et al, 2005; Kim et al, 2005). Intrinsic pathway can be reportedly initiated by e.g. the growth factor deprivation, oncogene activation and an excess of ROS. Moreover it has been proved that classical chemotherapy regimens (including DNA-damaging agents) often trigger an intrinsic pathway of apoptosis (Brenner et al, 2003). Undoubtedly the mitochondrion stands at the nexus of sensing and integrating diverse incoming stress signals, and mitochondrial disturbances often occur long before any marked morphological symptoms of apoptosis (Green, 2005; Skommer et al, 2007).

In recent years multiple mechanisms have been anticipated to explain mitochondrial function in cell death, including: release of apoptogenic proteins into the cytosol upon mitochondrial outer membrane permeabilization (MOMP) and loss of mitochondrial physiological processes indispensable for cell survival. Based on our current knowledge the MOMP is a fundamental event leading to a release of holocytochrome c (cyt c) and an array of death inducing small proteins (AIF, EndoG, Omi/HtrA2, Smac/DIABLO, Smac β) normally enclosed in the intermembrane space (IMS) of the organelle (Fig. 4) (Jiang and Wang, 2004; van Loo et al, 2002; van Gurp et al, 2003; Saelens et al, 2004). Although controversial, some authors postulate also the presence of pro-caspases (e.g. pro-caspase 3, 7, 8, 9) in the mitochondrial IMS which are being released during MOMP (Samali et al, 1999; Susin, et al 1999; van Loo et al, 2002; Garrido et al, 2006).

Dissipation of mitochondrial inner transmembrane potential ($\Delta\psi_m$) is frequently associated with MOMP. There are, however, examples of divergence where loss $\Delta\psi_m$ can precede, coincide or follow mitochondrial outer membrane permeabilization (Skommer et al, 2007). Interestingly, as described by us and others dissipation of mitochondrial inner transmembrane potential may not be an ultimate point of no return for cell commitment to die. In this regards it has been shown that in the absence of caspase activation some cells can reinstate mitochondrial inner transmembrane potential and/or eliminate damaged mitochondria (e.g. by activating autophagic

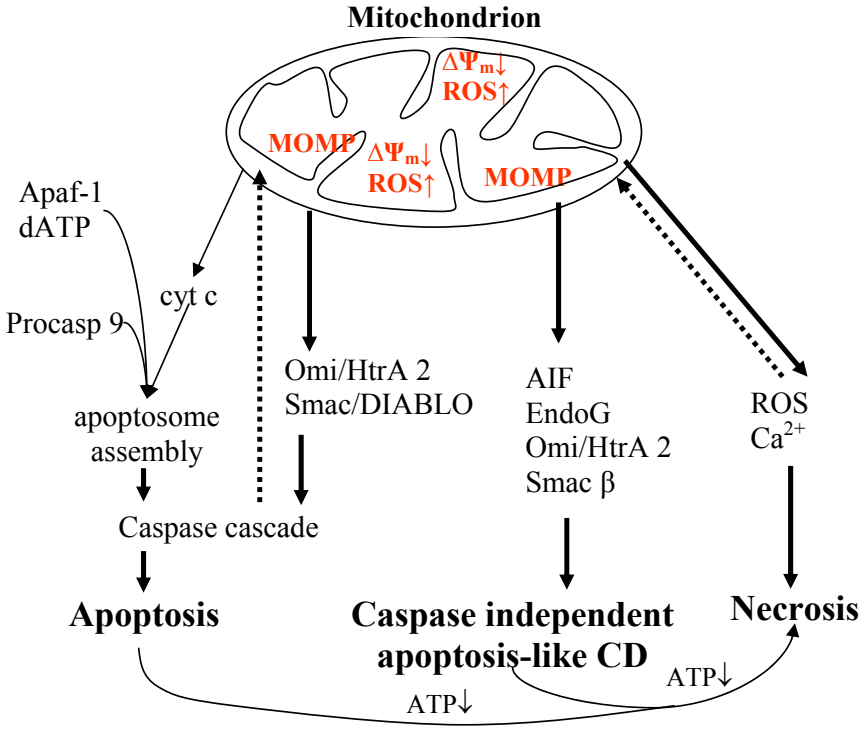
response) and re-establish their homeostasis (Milella et al, 2002; Wlodkowic et al, 2006).

Once MOMP is initiated, the discharge of cytochrome c from the IMS occurs. This subsequently leads to its binding to a cytosolic protease activating factor-1 (Apaf-1), followed by a conformational remodeling of Apaf-1, oligomerization and subsequent assembly of the apoptosome in the presence of dATP (Fig. 2, 4) (Hill et al, 2003). Apoptosome forms a molecular heptametrical mega-platform (responsible for the recruitment and activation of apical caspase 9 (Hill et al, 2003). After apoptosome assembly, pro-caspase 9 is massively enlisted by homotypic interactions between respective CARD motifs localized in the caspase regulatory prodomain and Apaf-1. Conceivably, the close proximity interactions between pro-caspase 9 molecules attracted to the apoptosome allow their dimerization, self-proteolysis and activation (Hill et al, 2003). Fully active caspase 9 subunits dissociate from apoptosome complex and participate in the cleavage of effector pro-caspases 3/7, hence initiating apoptotic cell dismantling (Fig. 2).

Interestingly the sole release of cytochrome c and formation of apoptosome is not always sufficient for executing cell demise. It has been demonstrated that the presence of endogenous inhibitor of apoptosis proteins (IAPs) and several heat shock proteins (HSP; e.g. HSP 27, 60, 70, 90) can amply impede both intrinsic and extrinsic pathways of apoptosis, often favoring malignant cell survival (Garrido et al, 2006). Owing to their importance in cancer development and resistance to therapy both groups attract, thus, mounting attention as potential anti-cancer targets (Reed, 2003; Reed and Pellecchia, 2005; Meng et al, 2006). IAPs are characterized by the presence of zinc finger-like bacculovirus inhibitor repeat (BIR) motifs and a C-terminal RING domain displaying E3 ubiquitin ligase activity. They are believed to thwart process of pro-caspase activation downstream of MOMP, inhibit activity of mature caspases and participate in ubiquitin-dependent proteasomal degradation of caspases. Therefore, the successful activation of caspases must be facilitated by the release of additional two small intermembrane space proteins: Smac/DIABLO and Omi/HtrA 2 that interfere with IAPs' function and allow seamless amplification of proteolytic cascade (Fig. 4) (van Gurp et al, 2003; Saelens et al, 2004).

Intriguingly, mitochondria are also reported as master regulators of caspase independent cell demise programs (Fig. 4) (Ferri and Kroemer, 2001; Cande et al, 2002; Lorenzo and Susin, 2004; Kroemer and Martin, 2005).

Fig. 4 Downstream effects of mitochondrial outer membrane permeabilization (MOMP) (based on: van Loo et al, 2002; van Gurp et al, 2003; Saelens et al, 2004)



It is generally believed that in response to some triggers, the MOMP permits the discharge of evolutionary conserved small intermembrane space proteins: apoptosis inducing factor (AIF) and/or Endonuclease G (EndoG) (Kroemer and Martin, 2005). Extensive exploitation of pharmacological caspase inhibitors, genetic *knock-out* and *knock-down* approaches and microinjection studies lead to the discovery of caspase independent cell killing mediated by those two IMS proteins. Intriguingly, there is presently an avalanche of data supporting the notion that apart form AIF and EndoG other mitochondrial IMS proteins might participate in initiation and/or execution of cell death programs under circumstances of diminished or abrogated caspase activity (Fig. 4) (van Loo et al, 2002; van Gurp et al, 2003; Lorenzo and Susin, 2004; Hail et al, 2006). It has been recently demonstrated that cytoplasmic overexpression of Omi/HtrA 2 effectively induces cell death solitary dependent on its intrinsic serine protease activity (Suzuki et al, 2001). IAPs interaction motifs of the molecule were not essential in executing cell dismantling which proceeded in a caspase independent manner (Faccio et al, 2000; van Loo et al, 2002; Lorenzo and Susin, 2004). Moreover,

Smac β a recently described splice variant of Smac/DIABLO, lacking IAPs binding capability, exhibits considerable pro-apoptotic properties (Roberts et al, 2001). Although Smac β-mediated cell demise is poorly understood similar effects have been induced by an overexpression of truncated form of Smac/DIABLO, totally lacking its IAPs binding motifs (Roberts et al, 2001; van Loo et al, 2002). Thus, Omi/HtrA 2 and Smac/DIABLO emerge nowadays as double edge swords capable not only to antagonize IAPs function and promote caspase dependent apoptosis but possibly also to complete cell dismantling when caspase activation is pathologically abrogated. Topical discoveries by Vandenabeele's group shed also light on possible role of FABP (fatty acid-binding protein), ACBP (Acyl-Co-A-binding protein) and PTB (poly-pirimidyne track-binding protein) in caspase independent regulation of cellular demise. Those proteins were reportedly released from isolated mitochondria upon treatment with recombinant tBid (van Loo et al, 2002). Even though their direct link to the cell death apparatus is still elusive and requires further investigation, they provide further evidence on the fundamental role of mitochondria on the crossroads of cellular life and death.

Owing to the importance of the above studies for our understanding of cancer development and advance of future anti-cancer regimens it is of no surprise that mitochondria and its IMS molecules represent a scientific hot-topic during recent years. The precise decision making at the mitochondrial level, leading to different death phenotypes is, however, far from fully understood. In this context, it has been even controversially postulated that MOMP may be a selective and rate limiting step in response to diversified array of death triggers. Indeed, it has been reported by some authors that the release of small intermembrane space proteins may be a subject to tight regulation and, although debatable, selectivity of MOMP in various cellular scenarios cannot be completely ruled out. To the best of our current knowledge the mitochondrial pathway of apoptosis is synchronized upstream of MOMP by a tight regulatory network of Bcl-2 family proteins responsible for stress sensing and permeabilization of the outer mitochondrial membrane (Spierings et al, 2005, Skommer et al, 2007). Pertinent to the importance of Bcl-2 family proteins in cancer biology and anti-cancer therapy, those aspects will be briefly explored in the following chapter.

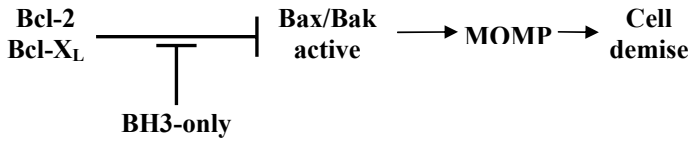
2.4 Mitochondria and the Bcl-2 family – dancing with devils

The seminal discovery that the *BCL2* gene inhibits cell death rather than promotes cell proliferation gave the foundation for a nowadays widely appreciated theory that impaired cell death is a decisive stage in a multi-step carcinogenesis (Bakhshi et al, 1985; McDonnell et al, 1989; Spurgers et al, 2006). Actually, oncogene activation often promotes apoptosis, and thus cancer cells can endure only if additional survival-promoting mechanisms are acquired (Kirkin et al, 2004; Spurgers et al, 2006). Following the pioneering work of Dr Stan Korsmeyer, the role of Bcl-2 family members in regulation of life/death switch and tumor progression has thoroughly been studied (Vaux et al, 1988; McDonnell et al, 1989; Nunez et al, 1990; Letai et al, 2004).

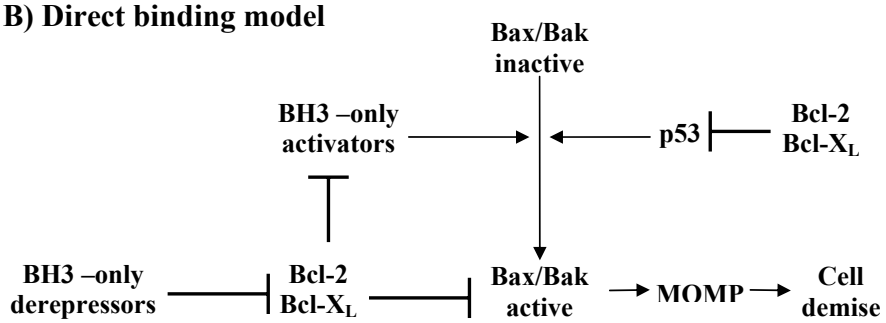
Bcl-2 family members are perceived as effective watchdogs of the mitochondrial changes during both apoptosis and necrosis (Scorrano and Korsmeyer, 2003; Green and Kroemer, 2004; Chipuk et al, 2006). Novel findings postulate also their emerging role in the regulation of autophagy (Pattingre and Levine, 2006). Moreover, although traditionally linked to the regulation of mitochondrial membrane permeability, awareness of their unexpected functions at the endoplasmic reticulum and in cell cycle regulation is rising (Thomenius and Distelhorst, 2003; Gross, 2006). Importance of Bcl-2 proteins in sustaining viability of malignant cells is frequently exemplified by picturesque descriptors such as “the lords of death”, “gatekeepers and gatecrashers” or “bodyguards and assassins”, etc (Fleischer et al 2003; Packham and Stevenson, 2005). Up to date approximately 30 members of the family have been identified. The Bcl-2 family can be conveniently classified based on both structural and functional criteria on: pro-survival proteins, containing all four Bcl-2 homology (BH) domains (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, A1, Boo) or pro-apoptotic proteins. The latter cluster is further separated into multi-domain pro-apoptotic Bcl-2 proteins (Bax, Bak and Bok) and BH3-only proteins (Bad, Bim, Bid, Noxa, Puma, and others). Multi-domain pro-apoptotic proteins share a considerable degree of structural resemblance to pro-survival Bcl-2-like proteins and contain BH1-3 domains. BH3-only proteins contain single BH3 domain responsible for protein-protein interactions (Danial and Korsmeyer, 2004; Green, 2005; Chipuk et al, 2006; Skommer et al, 2007).

Fig. 5 Mutual interaction between Bcl-2 family members: A) The displacement model - constitutively active Bax/Bak is constrained by pro-survival Bcl-2 family members, counteracted by BH3-only proteins. B) The direct binding model - Bax/Bak may be activated only upon interaction with BH3-only activators (Bid, Bim), and Bcl-2 anti-apoptotic proteins held this interaction in check until inhibited by de-repressor BH3-only proteins. The transcription-independent function of p53 as a direct activator is also highlighted. C) Specificity of BH3-only proteins. Note that only tBid, Puma, Bim interact with all anti-apoptotic Bcl-2 proteins. (comprehensively reviewed in Skommer et al, 2007).

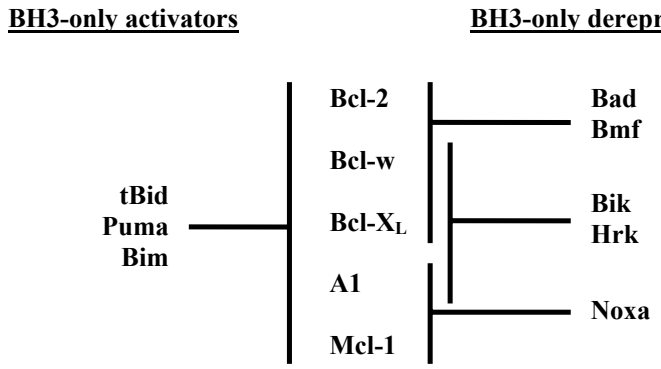
A) Displacement model



B) Direct binding model



C) Specificity of BH3 domains towards anti-apoptotic Bcl-2



The action of Bcl-2 family members is strongly linked to the molecular regulation of mitochondrial membrane permeabilization and release of cytochrome c (Danial and

Korsmeyer, 2004; Letai, 2005; Green, 2005; Chipuk et al, 2006). Although still far from fully understood the complex, regulatory network of Bcl-2 proteins is being gradually uncovered. The conservative model ("rheostat model") assumed that the anti-apoptotic Bcl-2 family members bind to the multi-domain pro-apoptotic members, and thus prevent them from mediating the release of cytochrome c from mitochondrial intermembrane space (Korsmeyer et al, 1993; Bouchier-Hayes et al, 2005; Letai, 2005; Skommer et al, 2007). The updated, contemporary model ("switched-rheostat model") implements the profound role of BH3-only molecules which conceivably exist in two classes: "de-repressors" and "direct activators" (Fig. 5 A, B) (Bouchier-Hayes et al, 2005; Letai, 2005; Skommer et al, 2007). In this model, the BH3-only proteins can presumably bind to the hydrophobic pocket on the anti-apoptotic Bcl-2 proteins, displacing activator BH3-only proteins (direct binding model), or the multi-domain pro-apoptotic family members (displacement model) (Fig. 5 A, B) (Bouchier-Hayes et al, 2005; Chipuk et al, 2006; Skommer et al, 2007). Moreover, the recent data strongly indicate that the specificity of particular BH3-only proteins towards anti-apoptotic members of the family seem to diverge, complicating further the complexity of Bcl-2 interactions (Fig. 5 C) (Letai, 2005; Skommer et al, 2007). The final outcome of both binding models is initiation of MMP, release of cytochrome c, the array of previously described small intermembrane space molecules and initiation of apoptotic cell demise (van Gurp et al, 2003). Contemporary models concentrate mostly on the extensive range of molecular interactions between Bcl-2 family proteins that occur upstream of mitochondria. The direct Bcl-2-mediated control of mitochondrial membrane stability is yet another scientific puzzle.

According to existing theories Bcl-2 family members can:

1) insert into OMM, oligomerize, and form membrane-spanning pores
2) interact with and regulate pre-existing channels such as permeability transition pore (PTP) or mitochondrial apoptosis-induced channel (MAC)
3) temporarily alter the membrane curvature by interactions with membrane lipids
4) together with heat-shock proteins regulate formation of pores from conglomerates of misfolded proteins in the OMM (Lemasters et al, 2002; Newmeyer and Ferguson-Miller, 2003; Skommer et al, 2007).

As those mechanisms are often cell line and stimuli dependent it will probably take some time until we will fully comprehend the mechanistic fundamentals of MMP.

2.5 Inter-organelle cross-talk in sensing and executing cellular dismantling

The thorough understanding of the intrinsic and extrinsic pathways of apoptosis allowed gaining remarkable insights into the role and regulation of physiological and pathological cellular fates. The spectacular discoveries of Bcl-2 family members in initiation, cytochrome c/Apaf-1 in amplification and caspases in execution of cellular dismantling delivered, however, additional scientific riddles. The mushrooming studies using pharmacological inhibitors and genetic approaches provided doubts about the absolute universality of the mitochondrial pathway of apoptosis in response to many traumatic signals. Undoubtedly the fascination of a scientific community in the mitochondrion as a central hub controlling cell decease eclipsed for some time the crucial importance of other intercellular compartments. It soon became apparent, however, that the inhibition of caspases, overexpression of Bcl-2/IAPs, *knock-out or knock-down* of Bax/Bak/Apaf-1 does not always protect cells from dying (Rao et al, 2002). Thus, the critical query can be raised: if the mitochondria have a dual physiological function what about other intracellular organelles? Does mitochondrion really initiates and propagates all intrinsic death signals or it only represents a simple cog in a more complex intracellular continuum? Since biological systems generally show plasticity and redundancy: what kinds of countermeasures are activated when mitochondrial pathway is abrogated but cell still is capable of committing suicide?

2.5.1 Nucleus

Indisputably, the nuclear mechanisms regulating cell demise form the best studied examples of inter-organellar communications. The research in those aspects has been mostly driven by their enormous significance in our understanding of DNA-damage responses to the anti-cancer therapy (Crighton and Ryan, 2005). The seminal discoveries of p53 protein and further elucidation of transcription-dependent and transcription-independent functions of p53 delivered strong evidence of straight "conversation" occurring between nucleus and mitochondrion (Vogelstein et al, 2000). Although p53 is involved in many cellular reactions like: cell cycle control, genome integrity maintenance, DNA repair, etc it is also considered as a watchdog of apoptotic cell death (Schuler and Green, 2001; Crighton and Ryan, 2005). In this context the DNA-damage responses, sensed by the DNA-PK, ATM and ATR protein,

are transduced downstream through Chk1 and Chk2 enzymes to the p53 and E2F1 transcription factor (Fig. 6). The phosphorylation of serine residues at the N-terminal region of p53 leads to its stabilization and profound enhancement of trans-activation function (Ashcroft et al, 1999; Crighton and Ryan, 2005). A considerable number of apoptosis-related genes have lately been reported to be a transcriptional p53 targets (Schuler and Green, 2005). In this regard Bax, Puma and Bid proteins are among main targets that directly influence the mitochondrial pathway of apoptosis (Fig. 6) (Miyashita and Reed, 1995; Nakano and Vousden, 2001). The evidence exists that p53 can also trans-activate BH-3 only derepressor protein Noxa and a member of the apoptosome scaffold Apaf-1 (Oda et al, 2000; Moroni et al, 2001). Furthermore, p53 reportedly possesses trans-repression function that down-regulates expression of anti-apoptotic Bcl-2, Bcl-X_L and IAP proteins (Schuler and Green, 2001). Also a p53-regulated apoptosis-inducing protein 1 (p53AIP1), newly described p53 transcriptional target, presumably locates within mitochondrial matrix and induces mitochondrial membrane depolarization and apoptosis when over-expressed (Fig. 6) (Oda et al, 2000).

Imaginably the p53-dependent over-expression of Bax and down-regulation of Bcl-2 can, apart of mitochondria, directly influence endoplasmic reticulum (Fig. 6). In support of this hypothesis, Bax overexpression has been recently found to induce Ca^{2+} mobilization from ER stores whereas convincing evidences indicate that Bcl-2 co-localizes with ER and protects cells from thapsigargin-mediated apoptosis (Orrenius et al, 2003; Scorrano et al, 2003). p53 can thus supervise a deadly inter-organelle crosstalk, delivering further substantiation to the complex role of nucleus-ER-mitochondria network in initiation and propagation of programmed cell suicide.

Fascinatingly, recent discoveries shed surprising light on the transcription-independent role of p53 in apoptosis (Chipuk et al, 2003; Chipuk and Green, 2003). In this context, p53 has been reported to directly translocate to mitochondria and to interact with pro- and anti-apoptotic Bcl-2 family members acting as an enabler and de-repressor protein, respectively. (Chipuk and Green 2003; Chipuk and Green, 2004; Skommer et al, 2006). Thus, the p53 protein seems to present analogical properties as earlier described BH3-only proteins. Conceivably the transcription-independent action of p53, generate an initial wave of cellular response to stress, backed subsequently by a secondary, transcription-dependent mobilization of e.g. Bax, Puma and Noxa. This model of p53 vs. Bcl-2 crosstalk has been extensively reviewed by us and others

(Chipuk and Green 2003; Chipuk and Green, 2004; Skommer et al, 2007). In conclusion p53 lures as an evolutionary conserved, multifunctional cog sitting on the crossroad of life and death. It is feasible, however, that because of its fundamental function further, yet unexpected p53's associations are to become apparent in the forthcoming years.

It is noteworthy to highlight at this point that although widely appreciated, p53 is not a sole tumor suppressor acting in response to oncogenic stimulation and DNA damage. Recent discovery of the close p53 homologues (p63 and p73) allowed the recognition of p53-p63-p73 regulatory axis that complicated event more the overwhelming complexity of the tumor suppressor's network (Muller et al 2006; Pluta et al 2006). Lately revealed homologues use numerous promoters to produce a display of splicing forms. They are implicated both in normal tissue development and in tumor formation (DeYoung and Ellisen, 2007). P63 and p73 reportedly play an important role in apoptosis initiation by, similarly to p53, transactivating pro-apoptotic (e.g. CD95, TNFR1, TRAILR1, Bax, PUMA, Apaf-1) and transrepressing anti-apoptotic genes (e.g. HSP-70) (Muller et al 2006). Although still controversial, the mushrooming evidence indicates important roles of both homologues in progression of human malignancies and their responses to the anti-cancer therapy (Muller et al 2006; Pluta et al 2006). Interestingly, p63 and p73 isoforms come in many flavors including full length and N-termini truncated forms, which can act as transactivators of downstream targets or dominant negative repressors, respectively.

The ΔN-isoforms of p63 and p73 are postulated to have substantial oncogenic potential and contribute to cancer chemoresistance (Muller et al 2006). Current in vivo data suggest that p53 homologues may not function as conventional tumor suppressors as they are rarely mutated in human cancers and p73 deletion does not accelerate tumorigenesis in murine models (Perez-Losada et al 2005, Muller et al 2006). Likely it is the composition of different isoforms present in specific tissue context and unique tumor microenvironment signals that shape the dependence of different strains of the p53-p63-p73 regulatory axis (Muller et al 2006).

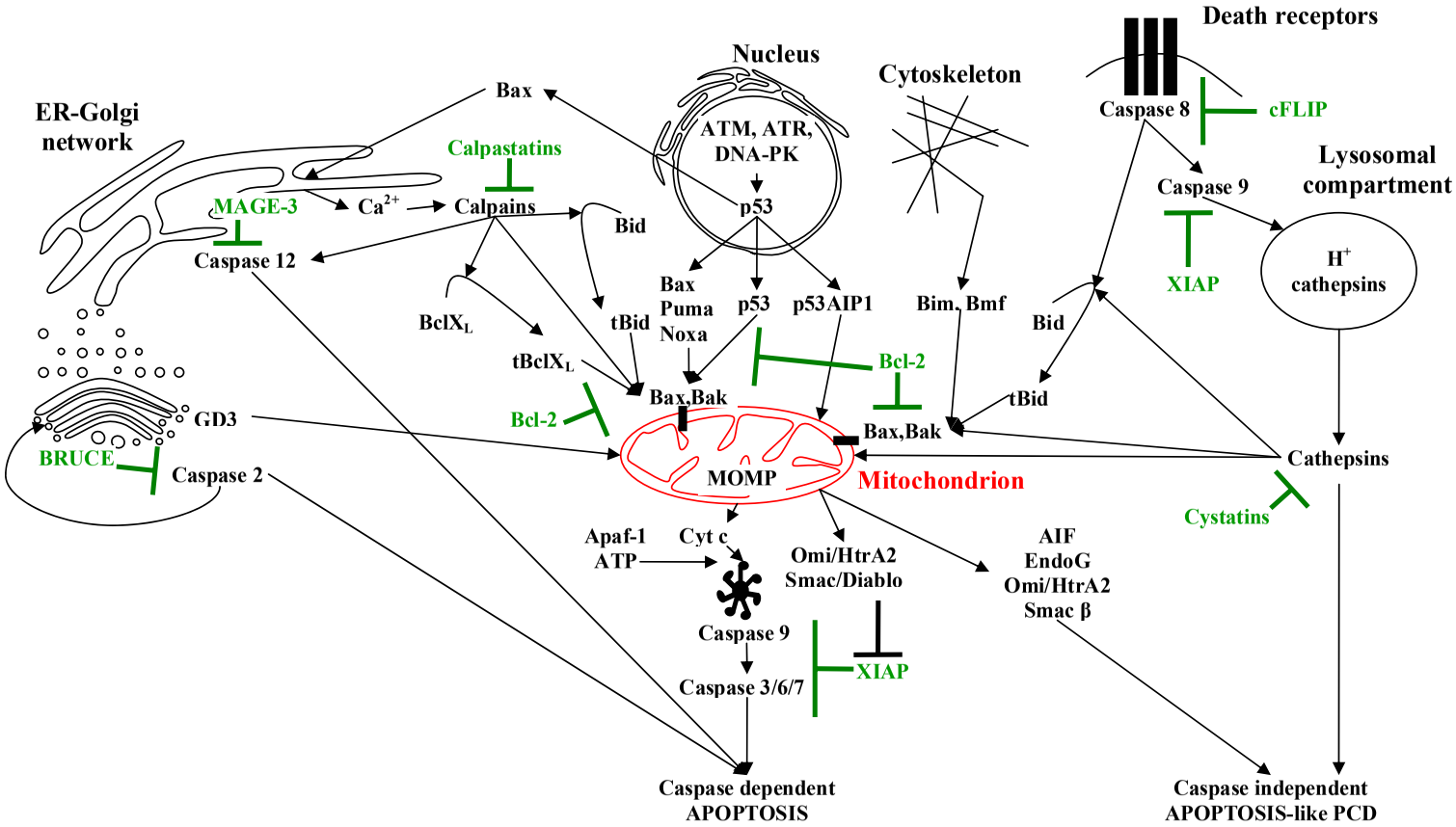
Aiming at the therapeutic implications of the p73 gene, the group led by Dr Kevin Ryan (Beatson Institute for Cancer Research, Glasgow, UK) has only recently reported a novel p53-derived apoptotic peptide (37AA hybrid peptide) that by de-repressing p73 from its negative regulator iASPP induces p73-dependent cell death *in vitro* and tumor regression *in vivo* (Bell et al 2007). Of note, 37AA-evoked cell death

was independent of the p53 status and selective to malignant cells (Bell et al 2007). It is of particular importance as nearly 50% human tumors feature loss or mutations of p53, yet frequency of human p73 mutations appears to be extremely low. Thus, there is a substantial hope that by reinstating the pro-apoptotic function of p73 we will be able to robustly eradicate p53 mutant/p73 WT tumors (DeYoung and Ellisen 2007; Bell et al 2007).

2.5.2 Secretory pathway

For decades our knowledge on the pro-survival physiological functions of endoplasmic reticulum, Golgi apparatus, endosomal/lysosomal compartment, cytoskeleton and plasma membrane intensively evolved. We gained profound insights into the dynamic interaction between e.g. endoplasmic reticulum-Golgi apparatus-lysosomal-endosomal compartments and their direct structural/functional complexity in the rigid inter-organelle crosstalk required for cell endurance. Yet the assumption that every cell organelle is conceivably capable of sensing, amplificating and executing cell death is still a relatively novel and unexplored conundrum (Ferri and Kroemer, 2001; Maag et al, 2003; Momoi, 2004; Hicks and Machamer, 2005; Szegezdi et al, 2006). As recently shown by many authors, the secretory pathway is actively involved in sensing, launching and executing cellular demise (Fig. 6) (Maag et al, 2003). Moreover the evidence exists that decision making at the level of endoplasmic reticulum and Golgi apparatus may activate both pro-survival (recovery) mechanisms and, if the traumatic threshold is exceeded, cell suicide programs (Maag et al, 2003; Hicks and Machamer, 2005). The intense protein trafficking between ER-Golgi-endosomal/lysosomal compartments conceivably allows precise damage sensing and subsequent signal integration (Ferri and Kroemer, 2001; Maag et al, 2003). Together with the elements of secretory pathway, the mitochondrion and members of the Bcl-2 family often participate in integrating incoming death messages. The evidence is also mounting that signals initiated at the ER-Golgi network may possibly bypass mitochondrion and directly participate in carrying out the finely orchestrated cellular dismantling (Fig. 6) (Maag et al, 2003; Machamer, 2003).

Fig. 6 Simplified representation of the inter-organellar network responsible for sensing, amplification and executing of diverse cell demise programs. Mitochondrion is shown in red. Cell death promoting pathways are shown in black whereas cell death suppressor mechanisms are depicted in green (simplified and based on: Ferri and Kroemer, 2001; Maag et al, 2003; Jäättelä, 2004; Kim, 2005).



Endoplasmic reticulum (ER)

The involvement of endoplasmic reticulum in stress sensing encompasses two mainstream mechanisms: unfolded protein response (UPR) and calcium signaling (Fig 6, 7). UPR can be further divided into a transcription-dependent and transcription-independent mechanisms; the former heavily involved in initial cell recovery pathways (Ferri and Kroemer, 2001; Rutkowski and Kaufman, 2004; Walter and Hajnoczky, 2005).

Transcription-dependent response in initiating cell dismantling

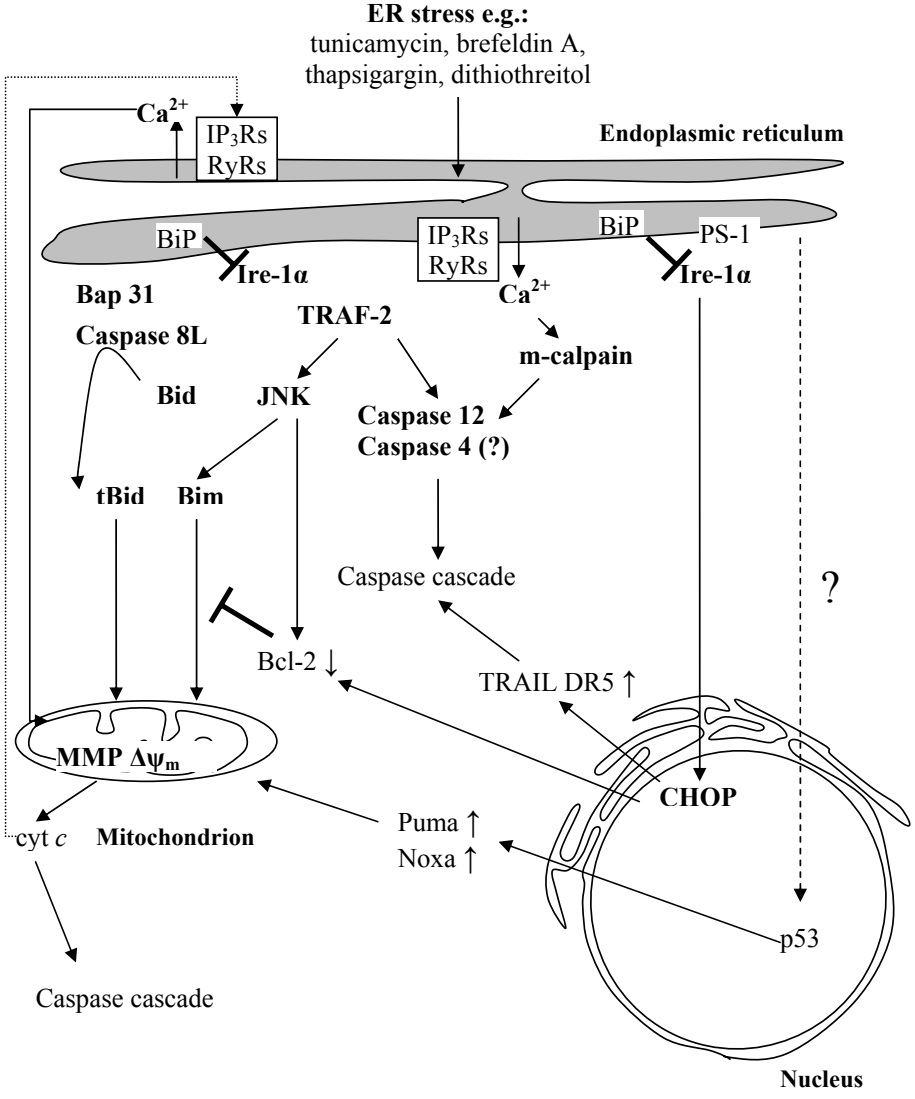
The accumulation of unfolded proteins in response to traumatic stimuli (e.g. inhibition of N-linked glycosylation by tunicamycin; disruption of ER-Golgi vesicular transport by brefeldin A or deficient formation of disulfide bonds by dithiothreitol) leads to their competitive binding to ER-luminal chaperon BiP/GRP78 (Bertolotti et al, 2000; Rutkowski and Kaufman, 2004). It displaces BiP/GRP78 form the ER-membrane residual serine-threonine kinase Ire1-α, allowing its dimerization and autophosphorylation on a cytosolic tail (Fig. 7). Following activation, the cytosolic fragment of Ire1-α undergoes cleavage by preselin-1 (PS-1) and translocates to nucleus where it promotes transcription of endoplasmic-resident chaperones (BiP, calreticulin) and transcription factor CHOP/GADD 153 (Fig. 7) (Katayama et al, 1999; Patil and Walter, 2001; Ferri and Kroemer, 2001). In the context of cellular demise CHOP/GADD 153 has been shown to down-regulate Bcl-2 expression and thus initiate mitochondrial apoptotic program (Fig. 7) (McCullough et al, 2001). Moreover, tunicamycin-induced ER-stress has recently been found to up-regulate expression of TRAIL receptor DR5 through CHOP/GADD 153 transcription factor in human prostate and carcinoma cells (Shiraishi et al, 2005). Intriguingly, the p53-mediated trans-activation of Puma and Noxa in response to thapsigargin and tunicamycin has been demonstrated in the recent work of Li and colleagues (Fig. 7) (Li et al, 2006). Although ER UPR-p53 link still remains elusive it delivers novel insights on the complexity of cellular signaling launched in response to secretory pathway stressors.

Transcription-independent response in initiating cell dismantling

Following activation, Ire1-α reportedly attracts adaptor protein TRAF-2 (TNFR-associated protein 2) (Yoneda et al, 2001). Formation of Ire1-α/TRAF-2 complexes at the cytosolic face of ER is postulated to activate c-Jun N-terminal kinase (JNK)

pathway and/or participate in recruitment, clustering and auto-activation of pro-caspase 12 molecules (Fig. 7) (Urano et al, 2000; Yoneda et al, 2001).

Fig. 7 Initiation of cell demise pathways in response to the ER stress (simplified and based on: Ferri and Kroemer, 2001; Momoi, 2004; Walter and Hajnoczy, 2005)



JNK reportedly inhibits Bcl-2 function leading to impaired sequestration of pro-apoptotic Bax, BH3-only proteins and regulation of ER Ca^{2+} stores (Bassik et al, 2004). There are also hints that JNK activity can directly modulate function of BH3-

only protein Bim, hence strongly influence mitochondrial-targeted pro-apoptotic signaling (Lei and Davis, 2003; Momoi, 2004; Szegezdi et al, 2006).

The discovery of the caspase 12 as an apical enzyme involved in response to ER damage delivered novel, speculative links to the classical apoptotic machinery (van de Craen et al, 1997). Although functionally present only in rodents, caspase 12, is localized specifically to the cytosolic side of endoplasmic reticulum (Nakagawa et al, 2000). Its activation in mouse embryonic fibroblasts has been reported to exclusively follow ER-targeted stress stimuli like: brefeldin A, tunicamycin and thapsigargin (Momoi, 2004). Moreover, inhibition of caspase 12 activity by overexpression of its endogenous inhibitor MAGE-3 has been reported to diminish ER-stress mediated apoptotic response in mouse cells (Fig. 6) (Morishima et al, 2002; Maag et al, 2003). There are, however, still conflicting reports on the requirement of caspase 12 in the ER stress response (Nakagawa et al, 2000; Momoi, 2004). Apart from Ire1-α/TRAF-2-mediated auto-proteolysis, several alternative models of activation have lately been proposed for caspase 12, including: Ca^{2+}-dependent cleavage by m-calpain; CARD domain-mediated clustering on an undefined Apaf-1-like adaptor molecule; GRP78 mediated formation of caspase 7/caspase 12 dimers (Momoi, 2004). As humans lack functional caspase 12 the quest is still ongoing to decipher its exact human orthologue (Fischer et al, 2002; Momoi, 2004). In this context caspase 4 has been recently demonstrated to co-localize with and participate in ER-induced apoptosis (Hitomi et al 2004). Complicating the complexity of apoptotic pathways an isoform of pro-caspase 8, pro-caspase 8L, has also been found to be activated at the ER via interaction with Bap31 protein (Breckenridge et al, 2002).

Finally, stress signals culminating at the endoplasmic reticulum can trigger rapid Ca^{2+} release from luminal ER stores. This process is efficiently executed by the inositol-1,4,5-triphosphate receptors (IP_3Rs) or ryanodine receptors (RyRs) serving as Ca^{2+} channels (Fig. 7) (Orrenius et al, 2003; Walter and Hajnoczy, 2005). Both pro- and anti-apoptotic members of the Bcl-2 family have also been postulated in regulation of the ER Ca^{2+} homeostasis (Thomenius and Distelhorst, 2003; Chen et al, 2004; Walter and Hajnoczy, 2005). Moreover as mitochondria often show close proximity contacts with tubular ER network their interactions cannot be without an influence on the inter-organelle information transfer (Rizzuto et al, 1998; Mannella et al, 1998). In this regard, recent work of Boehning and colleagues illustrated novel insight into the ER-mitochondrion crosstalk at the very early stages of apoptosis

induction. Clearly the initial cytochrome c release may initiate the activation of IP_3Rs, Ca^{2+} discharge from ER and subsequent feedback on mitochondria in the shape of by PTP dependent MMP (Fig. 7) (Boehning et al, 2003; Walter and Hajnoczy, 2005). Apart from eliciting mitochondrial MMP, the rapid Ca^{2+} mobilization from ER lumen can also elicit activation of cytosolic Ca^{2+}-dependent proteases, calpains. They are being seemingly involved in both caspase 12 processing and sole propagation of cellular dismantling (Fig. 6, 7) (Nakagawa and Yuan, 2000; Guicciardi and Gores, 2003).

Golgi apparatus

It has been known for some time that Golgi apparatus is being dissembled during apoptosis in a manner similar to that observed during mitosis (Sesso et al, 1999). Apoptotic cleavage of structural proteins localized to the Golgi has recently been found necessary to dismantle complex Golgi structure into separate stacks of cisternal membranes. In this context the overexpression of non-cleavable forms of structural golgin-160, p115 and GRASP65 often delay Golgi separation during caspase dependent cell death (Mancini et al, 2000; Chiu et al, 2002; Lane et al, 2002; Maag et al 2003). Yet a concept that Golgi apparatus may actually be actively involved in initiation and/or execution of cellular demise delivers a completely new insight on the secretory pathway as a watchdog of cellular fate (Maag et al, 2003; Machamer 2003; Hicks and Machamer, 2005).

The seminal discovery of caspase 2 and its co-localization to the Golgi apparatus and the nucleus delivered initial suggestions that Golgi apparatus may not only be “a passive player” in apoptosis (Fig. 6) (Mancini et al, 2000). Although caspase 2 has also been found to participate in DNA damage responses it conjunction with PIDDosome complex, it is reasonable that its function may be bidirectional and depend heavily on the cellular compartmentalization (Tinel and Tschopp, 2004; Zhivotovsky and Orrenius, 2005; Hicks and Machamer 2005). Pertinent to this notion it is now generally believed that a pool of caspase 2 localized at the cytosolic side of the Golgi apparatus may participate in initiation of apoptotic cascade in response to secretory pathway stresses. In support of this concept specific caspase 2-dependent cleavage of golgin 160 at Asp59 site has been shown as a very early apoptotic event that preceded caspase 3-dependent cleavage of golgin160 and poly-ADP-ribose polymerase (PARP) (Mancini et al, 2000; Machamer 2003). Captivatingly, recent report by Machamer’s group provided further evidence that caspase-resistant mutant

golgin 160 can abrogate apoptosis induced specifically by ER stress and ligation of death receptors (Maag et al, 2005). Moreover, BRUCE (**b**aculoviral-IAP-**r**epeat-containing **u**biquitin-**c**onjugating **e**nzyme) an expected negative regulator of caspase 2 activity has been localized to the Golgi superstructure (Fig. 6) (Hauser et al, 1998; Maag et al, 2003).

Besides caspase 2, the role of glycolipids and ceramides as messengers in death signalling pathways has also been raised during recent years. In the Golgi context, the activity of the GD3 synthase reportedly converts ceramide to the gangliosyde GD3. The latter has been shown to translocate to the mitochondrion and solely trigger MMP (Fig. 6) (Rippo et al, 2000; Malisan and Testi, 2002). Interestingly the suppression of GD3 synthase activity or its retention in the ER lumen markedly inhibits apoptosis (Tomassini et al, 2004). Moreover there are hints that mitochondrial GD3 targets are under rigid control of anti-apoptotic Bcl-2 family members (Malisan and Testi, 2002). Similarly to the GD3, a semilysobisphosphatidic acid has been found to shuttle from the Golgi to mitochondria in response to death receptor stimulation, supporting the significance of inter-organelle lipid signalling pathways (Cristea and Degli Esposti, 2004).

It is also conceivable that a pool of death receptors that populate Golgi apparatus in the normal physiological conditions demonstrate yet another link between Golgi and initiation of cellular demise (Ferri and Kroemer, 200; Maag et al, 2003). Some reports indicate that DRs may be quickly mobilised and shuttled to the cell surface during in response to p53 or GD3 signalling (Bennett t al, 1998; Maag et al, 2003). Moreover it is tempting to speculate that during apoptotic Golgi disassembly a surge release of DRs occurs which amplifies further the death cascade.

Not surprisingly Golgi apparatus lures as the upstream controller of many destruction cascades. The active protein transfer between ER-Golgi requires precise control mechanisms that as such are supposedly very sensitive to every pathological alteration in cellular homeostasis (Hicks and Machamer, 2005). Moreover the stability of steady-sate Golgi configuration is achieved not only by a subtle balance between antegrade vis-à-vis retrograde membrane trafficking but also by the specialized structural proteins like golgins and GRAPs involved in vesicle tethering and docking interactions between separate Golgi stacks (Hicks and Machamer, 2000). Cleavage fragments of golgins have been demonstrated to contain nuclear targeting motifs,

implicating further their role in the inter-organelle signal transduction (Mancini et al, 2000; Hicks and Machamer, 2002; Chiu et al, 2002). Furthermore, as postulated recently, Golgi is in fact enclosed in a highly specialized exoskeleton vigorously implicated in membrane anchoring, regulation of substrate diffusion and even enzyme positioning (Hicks and Machamer, 2005). It is plausible to speculate that unique Golgi exoskeleton may take part in integrating a plethora of death stimuli engaging from other parts of the cytoskeleton. Although speculative, the unique structure exoskeleton may have evolved to sense any distressing changes in the Golgi cisternal structure. In this regard any pathological physical abnormalities like: cisternal swelling, unstacking, alterations in membrane curvature or even thickness in Golgi membranes could hypothetically initiate recruitment of specific adaptor molecules that transduce cell recovery or cell demise signals (Hicks and Machamer, 2005).

Finally, Golgi apparatus is conceivably equipped with effective UPR sensing mechanisms compulsory to remove any misfolded or mutated proteins that escaped aforementioned ER-UPR system (Arvan et al, 2002; Hicks and Machamer, 2005). Therefore any overload of misfolded proteins reaching Golgi may set off initial cell recovery mechanisms and when they prove to be ineffective also cell demise pathways. Keeping in mind the crucial physiological position of this organelle it is tempting to speculate that, similarly to mitochondrion and endoplasmic reticulum, Golgi is poised to sense and integrate diversified death signals (Hicks and Machamer, 2005). Although the precise signalling pathways are still elusive it is unlikely that we will need to wait long for these questions to be answered.

2.6 Selective targeting of cell demise pathways in cancer therapy

Thanks to seminal discoveries in recent years it now becomes apparent that extensive inter-organelle crosstalk that regulates orchestrated cell dismantling is present in every cell type. Even if mitochondrion reportedly stands at the nexus of controlling cell fate in many models, redundant and failsafe mechanisms commencing from other organelles do exist and may in many cases efficiently override the inhibition of classical pathways. In this context, the importance of the organelle specific initiation of cell death signals is profoundly highlighted in cancer research field where the quest to override the intrinsic resistance of malignant clones to

apoptosis has direct links to the treatment outcome (Ferri and Kroemer, 2001; Kitada et al, 2002; Packham and Stevenson, 2005; Skommer et al, 2006).

Undoubtedly the appreciation of the death receptor pathway and recognition of the central role of mitochondria/Bcl-2 family members in the regulation and propagation of cell demise has uncovered novel targets for anti-cancer therapies (Pellecchia and Reed, 2004; Reed and Pellecchia, 2005; Fantin and Leder, 2006).The alternative cell death pathways involving until recently underappreciated organelles such as endoplasmic reticulum, Golgi apparatus and lysosomes are, however, beginning to mushroom (Ferri and Kroemer, 2001; Jäättelä, 2004; Hicks and Machamer, 2005).

Thus, the present hope prevails that exploration of novel, non-redundant, fail-safe pathways of cell destruction might prospectively provide effective means to override cancer chemo-resistance (Anether et al, 2003; Linder and Shoshan, 2005; Kroemer and Jäättelä, 2005; Kim et al, 2006; Carew et al, 2006). The selected apoptosis based anti-cancer strategies with importance to the present thesis will be shortly discussed below.

2.6.1 A one shot kill - death ligands in cancer therapy

The opportunity of directly activating apoptotic program in malignant cells attracted considerable interest since the discovery of death receptor pathway. Originally, the FasL and TNFα ligands have been shown to possess remarkable *in vitro* anticancer properties against a wide panel of tumor cell lines and primary, patient derived samples. Although initial prospects seemed optimistic subsequent reports, utilizing *in vivo* models, proved that both ligands lack specificity and are excessively toxic to normal tissues (Meng et al, 2006; Ricci and Zong, 2006). Substantial side effects including hemorrhagic lesions, septic shock and hepatic failure in animal models excluded, thus, their further systemic administration (Ricci and Zong, 2006). Interestingly, TNFα found its restricted, investigational application for treatment of melanoma in isolated limb perfusion system (Ricci and Zong, 2006).

Although disappointing from the clinical perspective, the results achieved on Fas and TNF systems boosted further research on the therapeutic exploitation of death receptor pathway. The year 1996 marks the independent discovery of APO2L/TRAIL (APO2 ligand/TNF-related apoptosis-inducting ligand) by groups at the University of Michigan and biotech company Human Genome Sciences (HGS) in Rockville (Garber, 2005; Ricci and Zong, 2006). Extensive studies on TRAIL and its 5 receptors

(refer to the chapter 2.3.1 and Ashenazi, 2002) have delivered novel premises for DRs targeted therapies as cytotoxicity of TRAIL has reportedly been limited to tumor cells. Because TRAIL targeted therapies have potential to spare normal cells while inducing massive apoptosis in malignant cells they have recently been approved by FDA for clinical trials in patients with: advanced solid tumors, colon cancer, NSLC and NHLs (Meng et al, 2006).

Current development of effective medicines is funneled into two separate concepts: 1) utilization of stable, recombinant TRAIL ligand (led by Genentech – Amgen collaborative partnership; currently in phase 1 clinical trails) and 2) development of agonistic (activating) monoclonal antibodies against both DR4 and DR5 receptors (led by Human Genome Sciences; antibodies HGS-ETR1, HGS-ETR2 currently in phase 1/2 clinical trails) (Garber, 2005; Duiker et al, 2006; Ricci and Zong, 2006). Both concepts are being developed as single agent regimens and as a combinatorial approach with contemporary cytotoxic drugs (Ghobrial et al, 2005; Duiker et al, 2006).

The main impediment of the DRs based therapies is associated with reported resistance to TRAIL in majority of solid tumor cell lines (Meng et al, 2006; Duiker et al, 2006). Therefore, there is currently a substantial interest in modulation of DRs action and development of combinatorial treatment scenarios weakening or overriding tumor TRAIL-resistance (Meng et al, 2006). Moreover as the physiological functions of TRAIL still remain elusive there are unknown risk factors which have to be included into the ultimate therapeutic equation (Diehl et al, 2004; Garber, 2005).

2.6.2 Targeting Bcl-2 family members

As oncogene activation often promotes cell demise, cancer cells can survive only if they counterbalance intrinsic death signals by e.g. inactivation of p53-dependent pathway or over-expression of anti-apoptotic Bcl-2 family members (Spurgers et al, 2006). In this context, over-expression of Bcl-2, Bcl-X_L and/or Mcl-1, or loss of Bak and/or Bax function, has been suggested to contribute to tumor resistance to a wide array of conventional treatment protocols (Cory and Adams, 2005; Spurgers et al, 2006). Overexpression of BCL-2 gene is found in most follicular lymphoma, chronic lymphocytic leukemia and a quarter of large B-cell non-Hodgkin lymphomas. Also solid tumors including: prostate cancer, breast cancer, small cell lung carcinomas and non-small cell lung carcinomas, melanoma and gliomas feature pathological

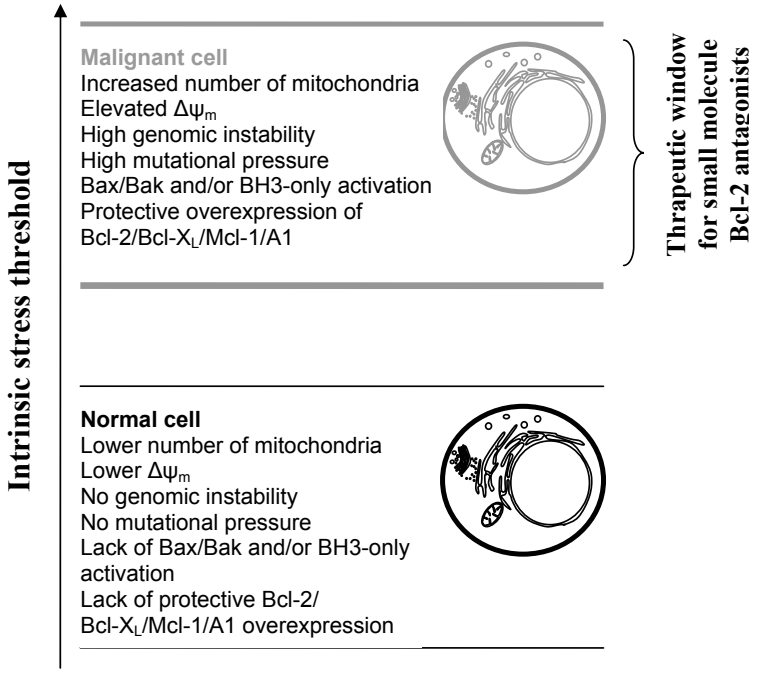
overexpression of BCL-2 related genes (Letai et al, 2004). Although defects in the ability to initiate cell demise program have been linked to cancer development and correlated with chemotherapy failure, cancerous cells feature a unique "Achilles´ heel". As the core components of their death machinery always remains intact they are intrinsically "primed to death" by intrinsic genomic instability, violation of cell cycle checkpoints, anoikis, etc, which are all postulated to activate pro-apoptotic Bcl-2 family members (Fig. 8) (Cory and Adams, 2005; Certo et al, 2006; Del Gaizo Moore et al, 2007). According to this theory, malignant cells are being "addicted" to prosurvival lesions and therapeutic agents able to restore cell demise machinery are expected to selectively eliminate malignant cells while sparing their normal counterparts not "primed to decease" (Letai et al, 2004; Certo et al, 2006; Del Gaizo Moore et al, 2007). It is of no surprise then that the recognition of the central role of mitochondria and Bcl-2 family in the regulation of cell demise has in recent decade uncovered novel targets for anti-cancer therapies (Cory and Adams, 2005; Letai, 2005). The cornerstone for current anti-Bcl-2 therapies has been laid by the influential research of Dr Stan Korsmeyer and his "Bcl-2 rheostat" hypothesis postulated back in 1994 (refer to chapter 2.4 and Skommer et al, 2006). Especially the anti-apoptotic members of Bcl-2 family (Bcl-2, Bcl-X_L, Mcl-1, Bcl-W, Bfl-1 and Bcl-B) represent the core interest in the rational drug design (Adams et al, 2005; Letai, 2005; Reed and Pellecchia, 2005). Current attempts to overcome cytoprotective effects exerted by anti-apoptotic Bcl-2 proteins in cancer cells, include development of drugs: 1) silencing the Bcl-2 gene expression; 2) enhancing expression of endogenous antagonists of Bcl-2; 3) inducing mRNA degradation with antisense oligonucleotides and 4) directly targeting protein-protein interaction using small-molecule inhibitors (Reed and Pellecchia, 2005).

As a proof of principle, the most advanced drug, Bcl-2 antisense oligonucleotide (oblimersen sodium, G3139, Genesense) has been successfully approved for clinical trials in non-Hodgkin's lymphomas and chronic lymphocytic leukemia (Meng et al, 2006). The drug has been designed against first six codons of the Bcl-2 and reported to synergize with conventional chemotherapeutics (e.g. DNA cross-linking agents and glucocorticoids). The major disadvantages of the antisense strategy are, however, the slow degradation rate of the Bcl-2 protein (which necessitates a prolonged suppression of mRNA accumulation) and potential inflammatory responses. Unfortunately recent results from phase III clinical trials were disappointing. In

multiple myeloma study oblimersen sodium failed to improve progression free survival in combination with dexamethasone. Likewise unsatisfactory results were obtained in chronic lymphocytic leukemia and melanoma studies (Ghobrial et al, 2006). G3139 was also disqualified from therapeutic dose escalation due to substantial off-target effects resulting in e.g. thrompocytopenia (Ghobrial et al, 2006). Finally recent reports by Raffo et al (2004) and Benimetskaya et al (2004) provided evidence that G3139 exerts its cytotoxic potential even in Bcl-2 depleted cells, raising uncertainty regarding specificity of the drug.

Conversely to antisense strategy, small molecule, cell permeable inhibitors of anti-apoptotic Bcl-2 family members are believed to have a wider therapeutic window with reduced potential to initiate unfavorable immune response (Fig. 8).

Fig. 8 Mechanisms underlying expected efficacy of the small molecule Bcl-2 antagonists



The mechanism of Bcl-2 antagonist action is based on the disruption of protein-protein interactions between pro- and anti-apoptotic Bcl-2 family members (Cory and Adams, 2005; Letai, 2005). As modeling of early peptidic Bcl-2 antagonist was based on natural BH3-only domains they are, thus, commonly referred to as "BH3

mimetics". "BH3 mimetics" have the potential to bind to the hydrophobic cleft on the surface of anti-apoptotic Bcl-2 proteins and by displacing pro-apoptotic multidomain and BH3-only proteins initiate intrinsic program of cell demise (Letai et al, 2002). The proof of concept for Bcl-2 antagonists was initially provided by studies on recombinant BH3 domains derived from Bid, Bim and Bad (Letai et al, 2002; Walensky et al, 2004; Certo et al, 2006). Considerable attention is currently being attracted to natural as well as synthetic peptide and non-peptide inhibitors of the Bcl-2 family members, differing in their specificity, efficacy and attainability *in vivo* (Letai, 2005; Reed and Pellecchia, 2005).

Pertinent to the therapeutic potential, small molecule "BH3 mimetics" effectively induce apoptosis in diverse cell lines and human primary cells derived from malignancies bearing protective over-expression of anti-apoptotic Bcl-2 family members (Lickliter et al, 2003; Oltersdorf et al, 2005, Campas et al, 2006; Konopleva et al 2006). Mushrooming reports suggest single agent efficacy and enhancement of conventional chemo- and immunotherapy-induced apoptosis upon combination with small-molecule Bcl-2 inhibitors in a wide variety of malignant cells and *in vivo* xenograft models (Chauhan et al, 2006; Shoemaker et al, 2006). New protocols employing such revolutionary compounds are currently studied with the therapeutic intent and have entered preclinical and phase 1 clinical evaluation (Graber, 2005).

It should be noted, however, that the underlying mechanisms of Bcl-2 antagonists are still intensively studied in view of the complex regulatory network of apoptotic pathways governed by the Bcl-2 family members. Alternative hypotheses have begun to emerge during recent years, including the role of the Bcl-2 family members in regulation of endoplasmic reticulum controlled apoptosis, autophagy and interaction with the up to date unidentified adapters able to activate initiator caspases other then caspase-9 (Milella et al, 2002; Thomenius and Distelhorst, 2003; Pattingre and Levine, 2006). Moreover recent report by van Delft et al (2006) has delivered intriguing questions regarding target specificity of the majority of putative "BH3 mimetics". Furthermore mechanisms of resistance to novel "BH3 mimetic" ABT-737 have only been recently described by two independent groups (Konopleva et al, 2006; van Delft et al, 2006).

Taken together, the elucidation of delicate molecular balance between apoptosis and survival delivered basics for our understanding of the molecular pathomechanisms underlying cancer development and progression. Moreover, thanks

to the seminal work of Dr Stan Korsmeyer proteins from the Bcl-2 family may soon be exploited with the therapeutic intent in a wide range of still incurable malignancies. It is, thus, tempting to speculate that inhibitors of Bcl-2-like proteins are one of the most promising experimental anti-cancer therapeutics of the XXI century. Further studies are necessary to develop more potent and selective inhibitors of Bcl-2 and related proteins, and gain further insights into the "devil dance" played by the members of the Bcl-2 family (Skommer et al, 2006).

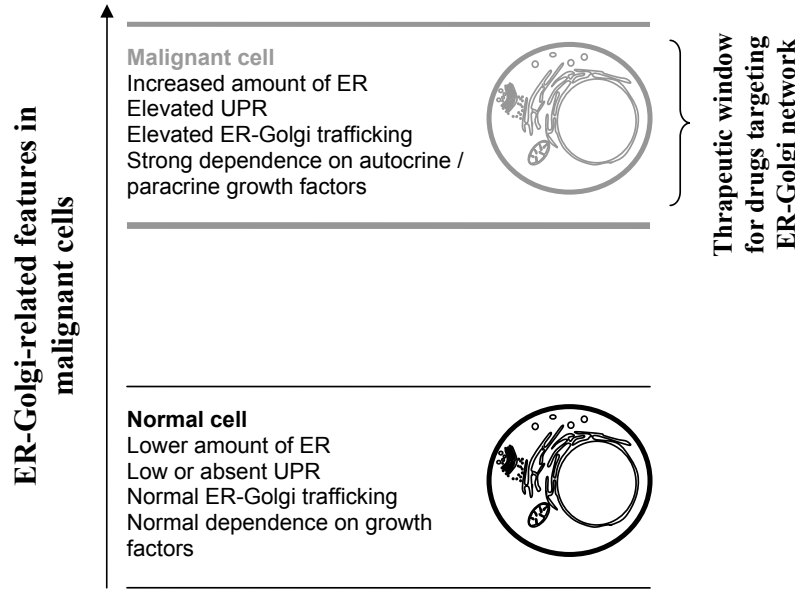
2.6.3 Engaging secretory pathway to combat malignancy

Although the burden of data indicates that elimination of hematopoietic malignant cells depends heavily on classical apoptotic pathways, the evidence is mounting that alternative apoptotic and non-apoptotic mechanisms may effectively contribute to tumor suppression (Ferri and Kroemer, 2001; Linder and Shoshan, 2006). A growing number of reports recognize endoplasmic reticulum and Golgi apparatus as key players in the sensing and execution of various death signals (refer to chapter 2.5.2). As the interest in the role of ER and Golgi during induction/execution of apoptosis has been gaining momentum, they simultaneously attract growing appreciation in the development of novel anti-cancer therapies (Ferri and Kroemer, 2001; Jäättela, 2004).

In this context, two recent studies indicated that malignant B lymphocytes feature not only increased mitochondrial content but also more elaborate endoplasmic reticulum (ER) network when compared to their normal counterparts (Carew et al, 2004; Carew et al, 2006). Therefore, it has been postulated that ER-Golgi system may be imperative for endurance of malignant B-clones, and such reliance may reveal unique anti-cancer targets (Fig. 9) (Carew et al, 2006).

Pertinent to the therapy of B-CLL, the successful targeting of the ER-Golgi pathway with Brefeldin A (BFA) in fludarabine refractory CLL cells has recently provided novel insights on how to eradicate cancerous B-cells independently of their p53 status and pathological overexpression of Bcl-2, Bcl-X_L, Mcl-1 and XIAP proteins (Carew et al, 2006). Brefeldin A, a fungal 16-membered macrolide isolated from *Penicillium brefeldianum*, exerts ER and Golgi stress *via* inhibition of ADP-ribosylation factor (ARF). Subsequent decline in coatamer proteins assembly leads to the disruption of ER-Golgi vesicular transport, Golgi collapse due to the imbalance in retrograde transport and induction of apoptotic cell demise (Donaldson et al, 1992; Carew et al, 2006; Cheung et al, 2006).

Fig. 9 Mechanisms underlying expected efficacy of the drugs targeting secretory pathway



Apart from B-CLL cells, BFA reportedly triggered apoptosis in multiple myeloma (U266, NCI-H929), Jurkat, HeLa, leukaemia (HL60, K562, BJAB), colon (HT-29), prostate and adenoid cystic sarcoma cells (Shao et al, 1996; Wallen et al, 2000; Salles et al, 2004; Boya et al 2002; Carew et al, 2006). Moreover, Brefeldin A has been reported to exert its anti-tumor activity in melanoma athymic mouse xenografts and enhance action of staurosporine and 7-hydroxystaurosporine in human promyelocytic leukemia cells (Sausavile et al, 1996; Shao et al, 1996). Importantly, a water soluble prodrug form of BFA, Breflate (NSC656202), is currently under development as a investigational anticancer agent that has shown promising pharmacokinetics in mice and beagle dogs (Phillips et al, 1993; Phillips et al, 1998).

Recent studies by Tinhoffer et al (2002) and Anether et al (2003) have also revealed that Tetrocarcin A, an antibiotic isolated from actinomyces, eradicated T-acute lymphoblastic leukemia (T-CLL) and B-CLL cells through ER mediated stress, independently of Bcl-2 status. Similarly to BFA, Tetrocarcin A efficiently initiated cell death in fludarabine refractory B-CLL primary cells (Anether et al, 2003). Of particular interest tunicamycin, another ER stress inducing antibiotic that blocks N-

linked glycosylation, reportedly sensitizes human prostate cancer cells to TNF-related apoptosis-inducing ligand (TRAIL) (Shiraishi et al, 2005). Furthermore, pertinent to the role of SERCA pump in ER mediated apoptosis, modified thapsigargin (PSA-activated thapsigargin) has recently been postulated as an investigational drug for prostate cancer (Denmeade and Isaacs, 2005). Finally, as an unfolded protein response (UPR) forms an important cytoprotective mechanism, a proteasome inhibitor bortezomib augmented tunicamycin- and thapsigargin-triggered apoptosis in pancreatic cancer cells (Nawrocki et al, 2005).

Taken together it is tempting to speculate that agents that disrupt the secretory pathway may represent a completely novel avenue in anti-cancer drugs development. Further studies are necessary to elucidate precise signalling pathways launched from ER-Golgi compartment and to develop more potent and selective drugs that activate ER-Golgi mediated cell demise in malignant cells.

2.7 Cytometry in studies of tumor cell demise

Undisputable advantage of FCM technology is pronounced by the fact of powerful multiparameter analysis capability and rapid analysis times that it offers (Herzenberg et al, 2002; Shapiro, 2003). Nowadays, bench-top cytometers and high-speed sorters are capable of analyzing up to 16 optical parameters from a single cell, with acquisition rates exceeding 25 000 events per second, and accuracy of individual cell measurement approaching 1%, opening completely new horizons for the cell biology research (Bernas et al, 2006). Moreover as flow cytometry allows single cell analysis, the heterogeneity of population is preserved and enables quantitative discrimination of discrete cell subpopulations (Darzynkiewicz et al, 2001). Thus, FCM overcomes common problems of traditional techniques like fluorimetry, spectrofotometry, etc., involving considerable averaging of the results from a given sample. Novel technologies like cell imaging in flow and laser scanning cytometers deliver even more sophisticated features that combine superior statistical power of cytometric analysis coupled with high-resolution imaging capabilities (Darzynkiewicz et al, 1999; Darzynkiewicz et al, 2001; Deptala et al, 2001; Smolewski et al, 2001; George et al, 2004).

As apoptosis is a complex, finely controlled process of great relevance in tissue homeostasis and pathogenesis it is of no surprise that cytometry found its noteworthy applications in cell death studies. The majority of classical apoptosis features can be

rapidly examined by both flow and image cytometry, that have proven to be reliable and flexible platforms in a wide spectrum of research and clinical applications (Darzynkiewicz et al, 1997; Halicka et al, 1997; Bedner et al, 1999; Smolewski et al, 2003; Huang et al, 2005). Analysis of cell demise modes using fluorescently labeled functional probes permits also cell sorting with subsequent supplementary biochemical and molecular studies. Importantly, multiparameter data obtained by flow and image cytometry permit ultimate correlation of different cellular events at a time on a cell-by-cell basis (Rasola et al, 2001; Pozarowski et al, 2003). Not surprisingly the development of novel functional probes for cell death studies and thorough understanding of the exact mechanisms underlying properties of existing ones are of utmost importance for the progress in cell necrobiology (Darzynkiewicz et al, 1997; Darzynkiewicz et al, 2001). This is particularly relevant in view of the growing appreciation of the multitude of cell demise modes, and the need for sensitive and high-throughput applicable assays capable to discriminate them.

In this context, patented by *Molecular Probes* SYTO probes are slowly gaining attention as relatively inexpensive, convenient to use and sensitive markers of apoptotic cell death (Frey, 1995; Poot et al 1997; van der Pol et al, 2003). SYTO dyes reportedly provide the means for tracking apoptosis in diverse cell lines, primary cells and have been proved amenable for the development of multiparameter flow cytometry assays (Frey, 1995; Poot et al, 1997; Schuurhuis et al, 2001). They exhibit very low inherent fluorescence, with strong enhancement upon binding to DNA/RNA. Moreover, the SYTO-stained eukaryotic cells exhibit both nuclear and diffuse-cytoplasmic staining pattern, the latter reportedly abolished after formaldehyde fixation (Broxterman et al, 1997; van Zandvoort et al, 2002; Mundy and Freudenrich, 2006). Of note, SYTO probes are not exclusive DNA stains, as they have been successfully applied to visualize translocation of RNA granules in neurons and discriminate nuclear/mitochondrial DNA from cytoplasmic RNA using two-photon lifetime imaging (Knowles et al, 1996; van Zandvoort et al, 2002). Importantly some authors postulate also the reliability of at least some SYTO dyes as effective substrates in quantitative P-glycoprotein function measurement (Broxterman et al, 1997; Schuurhuis et al, 2001; van der Pol et al, 2003).

Although mounting evidence show equal or higher sensitivity of SYTO probes as compared to Annexin V based assays, the precise mechanism underlying SYTO differential staining of apoptotic and viable cells still remains unclear (Schuurhuis et

al, 2001; Sparrow and Tippett, 2005). The most widely embraced idea is the self-quenching of SYTO molecules that follows changes in interprobe proximity during apoptotic chromatic condensation. Also the decrease in SYTO binding sites as the chromatin condensation and/or RNA degradation advances in the process of apoptosis has been postulated by some authors during recent years (Frey, 1995; Poot et al, 1997; Zandvoort et al, 2002). Finally, based on the observation that most of the SYTO dyes contain one net positive charge at neutral pH and therefore may resemble mitochondrial membrane potential (MMP)-sensitive probes, it has been also suggested that alterations in binding of SYTO to mitochondrial DNA or decrease in its $\Delta\psi_m$-driven mitochondrial uptake may contribute to the overall reduction of SYTO fluorescence in apoptotic cells (Broxterman et al, 1997; van Zandvoort et al, 2002; Sparrow and Tippett, 2005). These noteworthy but so far limited data clearly call for further investigation with aim to reveal mechanisms responsible for a reduction of SYTO fluorescence in apoptotic cells as compared to viable cells.

3. Objectives of the study

The specific aims of the project were to:

(i) Assess for the first time the monotherapeutic efficacy of novel small-molecule Bcl-2 inhibitor HA14-1 and evaluate its potential combinatorial treatment with conventional anti-cancer chemotherapeutics: dexamethasone, doxorubicin and vincristine in recently established follicular lymphoma (FL) cell lines. (**Article I**)

(ii) Track the multitude of apoptotic events, cell cycle specificity and temporal relationship between caspase activation, mitochondrial membrane depolarization and plasma membrane permeabilization in FL cells upon stimulation with Bcl-2 antagonist HA14-1. (**Article II**)

(iii) Assess for the first time the monotherapeutic potential of ER-Golgi transport disrupting agent Brefeldin A and follow up on the multitude of cell demise events using multiparameter flow cytometry in FL cell lines (**Article IV**)

(iv) For the first time assess the potential of simultaneous treatment using Brefeldin A and the small-molecule Bcl-2 inhibitor HA14-1 or CD95 cross-linking monoclonal antibody (**Article IV**)

(v) Provide novel insight into mechanisms underlying SYTO probes staining properties in different cell death contexts, applying state-of-the-art multiparametric flow cytometry, and multicolor fluorescent microscopy (**Article III**)

4. Materials and methods

4.1 Cells and culture

The origin and characteristics of human follicular lymphoma (FL) cell lines HF1A3, HF4.9 and HF28RA were as previously described (Eray et al, 2003). Cells were cultured in 100 ml (75 cm^2) cell culture flasks (Sarstedt Inc, Newton, NC, USA) in RPMI 1640 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 5% heat-inactivated FCS (EuroClone, Pero, Italy), 2 mM L-glutamine (Fluka Chemie, Buch, Switzerland), 200 µg/µl streptomycin (Cambrex), 240 IU/ml penicilin (Cambrex), 10 mM HEPES buffer (Cambrex), 0.1 mM NAA (Cambrex), 1 mM Na-pyruvate (Cambrex) and 20 µM 2-mercaptoethanol (Fluka Chemie). All cell cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere. During experiments cells were always in asynchronous and exponential phase of their growth.

Multi drug resistance efflux pump status of HF1A3, HF4.9 and HF28RA cells was evaluated by flow cytometry using cyclosporine A (Sigma-Aldrich Corp., St Louis, MO, USA), verapamil (Alexis Biochemicals, Lausen, Switzerland), probenecid (Alexis Biochemicals) together with SYTO 16 probe (Molecular Probes, Eugene, OR, USA) as previously described (Broxterman et al, 1997). P-gp-attributable activity was not detected in any of the cell lines.

4.2 Induction of apoptosis and primary necrosis

To induce apoptosis, follicular lymphoma cell lines were seeded on the 24 or 48-well flat bottom polystyrene cell culture plates (Corning Inc, NY, USA), treated with various concentrations of: a small molecule Bcl-2 inhibitor HA14-1 (Alexis Biochemicals; 0-25 µM), Brefeldin A (Sigma; 0-1000 ng/ml), CD95 cross-linking antibody (clone CH11; Upstate, NY, USA; 0-1000 ng/ml), dexamethasone (Dex; Sigma; 0-1000 nM), cycloheximide (CHX; Sigma; 0-10 µg/ml), doxorubicin (Dox; Sigma; 0-1000 nM) vincristine (Vin; Sigma; 0-100 nM) and harvested at different times as indicated in the respective articles. Small molecule Bcl-2 antagonist HA14-1 was dissolved in DMSO and, owing to its instability, stored at -70˚C in 1mM aliquots. A fresh aliquot was used for each experiment immediately after thawing. Due to light sensitivity of HA14-1 compound experiments were performed avoiding direct light

illumination. Brefeldin A was dissolved in DNA grade ethanol and aliquots were stored at -20ºC.

Primary necrosis was induced in HF1A3 cells by hyperthermia (56˚C; 5min or 46˚C up to 6h), treatment with 1% sodium azide (NaN_3; Sigma) for up to 4h, or 3% H_2O_2 for up to 45min.

In order to inhibit caspase-dependent cell death cells were pretreated for 2 hours with a pan-caspase inhibitor z-VAD-fmk (Calbiochem, Cambridge, MA, USA; 10-100 μM), a caspase 3/7 inhibitor z-DEVD-fmk (Calbiochem) and a caspase 9 inhibitor z-LEHD-fmk (Calbiochem) as described in the respective articles. Cathepsin and calpain inhibitors (Pepstatin A, MDL28170, PD150606, CA-074-Me, zFA-fmk, and ALLN) were generously provided by Dr M. Courtney (A.I. Virtanen Institute, Kuopio, Finland). All inhibitors were dissolved in DMSO and their aliquots stored at -20ºC. In some experiments reactive oxygen species (ROS) scavengers: N-acetylcysteine (NAC, Sigma) and ascorbic acid (AA, Sigma) were applied. Both compounds have been dissolved in PBS and their aliquots stored at -20ºC.

No loss of activity of any of the compounds was detected over the duration of the study. All compounds were diluted in complete cell culture medium to working stocks immediately before use. The final concentration of ethanol/DMSO in culture media did not exceed 0.2% (v/v), and no alterations in growth variables were detected in vehicle controls.

4.3 Cell proliferation assays

To assess short-term proliferation effects, cells were seeded on the 96-well flat bottom polystyrene culture plates at initial concentration 250.000 cells/ml and treated with 0 – 100 ng/ml BFA in complete medium for 20h. Subsequently 1 μCi/ ml [methyl-^{3}H]-thymidine was added for additional 4h incubation at 37ºC. The incorporated radioactive thymidine was quantified by scintillation counting with Microbeta counter (Perkin Elmer, Wellesley, MA, USA).

To examine long-term effects of BFA treatments, cells were seeded at initial concentration 100.000 cells/ml and treated with 0-75 ng/ml BFA for up to 5 days. At the indicated intervals, 20 μl cell samples were removed and viable cell number was assessed by standard Trypan Blue (Life Technologies, Grand Island, NY, USA) exclusion assay as previously described (Skommer et al, 2006).

4.4 MTT cytotoxicity assay

The cytotoxic effects of HA14-1 against follicular lymphoma cell lines were determined using the MTT assay ((3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) (Chemicon International, Temecula, CA, USA). Cells were seeded on the 96-well flat bottom polystyrene culture plates at initial concentration 5000 cells/well and treated with varying concentrations of HA14-1 (0-25 μM) for 20 h at 37˚C. Subsequently, the MTT solution was added and after 4 h of incubation the optical density (OD) was measured using microplate reader (Multiskan Plus, Labsystems, Finland). Extraction buffer was used as a blank control. The following formula was used to calculate cell viability: *cell viability (%) = (OD of the experiment samples/OD of the control)x100*. Sigmoidal dose-response curves were fitted to the mean cell viability plotted against log HA14-1 dose and lethal concentration 50% (LC_{50}) values were calculated from the resulting curves using Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA).

4.5 Flow cytometry and FACS sorting

4.5.1 Analysis

Flow cytometry analyses was performed on a FACScan (Becton Dickinson, San Jose, CA, USA) analyzer, equipped with 15 mW, 488 nm Argon-ion laser as a main excitation source and a standard setting of band-pass (BP) filters: FL1 (530/30 BP for collection of: SYTO green dyes, YO-PRO 1, FAM-VAD-FMK, FAM-DEVD-FMK, JC9 monomers and FITC-conjugated mAb fluorescence signals), FL2 (585/42 BP for collection of: propidium iodide, JC9 dimers, TMRM, NAO and DHE fluorescence signals), FL3 (650 LP for collection of: 7-AAD fluorescence signals). Acquisition of 10000 or 15000 events per each sample in 1024 channels resolution scale was done using CellQuest ver.3.3 software (Becton Dickinson) running under MacOS 8.1 operating system (Apple, Cupertino, CA, USA).

Analyses requiring double laser excitation setup were performed on an EPICS Elite ESP (Coulter Corp., Miami, FL, USA) cell sorter, equipped with 15 mW air-cooled Argon-ion laser operating at 488 nm excitation line and air-cooled He-Ne laser operating at 633 nm excitation line. Standard setting of band-pass (BP) filters was applied:

- 488 nm excitation line: FL1 (525 BP for collection of: SYTO16 fluorescence signals), FL2 (610 BP for collection of: propidium iodide fluorescence signals)
- 633 nm excitation line: FL4 (675 BP for collection of: Annexin V-APC fluorescence signals)

Acquisition of 10,000 events per each sample in 254 channels resolution scale was done using EPICS (R) Elite ver4.02 software (Coulter) running under DOS 3.07 operating system (Microsoft).

4.5.2 Cell sorting

Sorting of respective subpopulations was performed on EPICS Elite ESP (Coulter Corp., Miami, FL, USA) cell sorter using 488 nm Argon ion laser excitation line. Following band-pass filter configuration was applied: FL1 525 BP (SYTO16), FL2 610 BP (PI). Sort parameters and data acquisition were controlled by EPICS (R) Elite ver4.02 software (Coulter). Sort gates were drawn on bivariate FSC/SYTO16 dot-plots around three apparent subpopulations: $SYTO16^{high}$ (deemed viable cells), $SYTO16^{dim}$ (deemed early apoptotic cells), and $SYTO16^{low}$ (deemed late apoptotic/necrotic cells). Sorting was performed using 3x Sort-Sense Quartz Flow Cell (Coulter) with 100 μm diameter jetting orifice and crystal frequency set at 22 kHz.

To avoid destruction of sorted apoptotic subpopulations system pressure operated at 10 PSI and sort rates did not exceeded 3000 cells per second. All sorts were done using Purity1Recovery2 sort mode (Coulter) which allowed achieving ≥ 95% cell purity for each subpopulation. For subsequent fractional DNA content analysis at least 1×10^6 cells and for FSC/SSC analysis and sort purity check at least 5×10^5 cells were sorted into cooled RPMI 1640 medium containing 20% FBS. Each experiment consisted of at least three independent sorts.

4.6 Cell viability assessment

4.6.1 SYTO 16 / PI assay

The analysis was performed as previously described (Serafeim et al, 2003). Cells were exposed to selected agents at a dose and for the time indicated in the

respective articles. Control and treated cells were harvested, washed with PBS and resuspended in PBS containing 250nM SYTO 16 probe (Molecular Probes) and 5 µg PI. Following staining (20 min at RT in darkness) 500 µl of PBS containing 2% FBS (EuroClone) was added and cells were immediately analyzed on FACScan flow cytometer (Becton Dickinson). SYTO 16^{high} / PI^{neg} events were considered as viable cells.

4.6.2 YO-PRO 1 / PI assay

The analysis was performed as previously described (Idziorek et al, 1995). Cells were exposed to selected agents at a dose and for the time indicated in the respective articles. Control and treated cells were harvested, washed with PBS and resuspended in PBS containing 250nM YO-PRO 1 probe (Molecular Probes) and 5 µg PI. Following staining (20 min at RT in darkness) 500 µl of PBS containing 2% FBS (EuroClone) was added and cells were immediately analyzed on FACScan flow cytometer (Becton Dickinson). Double negative events (YO-PRO 1^{neg} / PI^{neg}) were considered as viable cells.

4.7 Evaluation of apoptotic features using flow cytometry

4.7.1 SYTO 16 / PI assay

The staining was performed as described in 4.6.1. Analysis was as previously described (Serafeim et al, 2003). Briefly, SYTO 16^{high} / PI^{neg} events were considered as viable cells, SYTO 16^{dim} / PI^{neg} events were considered as apoptotic cells and SYTO 16^{neg} / PI^{+} events were considered as late apoptotic/necrotic cells.

4.7.2 Comparative assessment of SYTO green probes

All probes from SYTO green I family (SYTO11-16) were procured from Molecular Probes. SYTO reagents were initially diluted in DMSO (Sigma) to achieve stock concentrations of 1 mM. Aliquots of probes were then stored at -20°C in the dark. After the treatment with apoptosis- or primary necrosis- inducing agents, cells were collected, rinsed with PBS to remove phenol red containing RPMI medium, and re-suspended in the 100 µl PBS containing selected SYTO dyes and plasma membrane permeability marker, propidium iodide (PI, Sigma, 5 µg/ml). Final

concentrations of SYTO dyes were as follows: 100 nM (SYTO11 and SYTO13), 200 nM (SYTO12), 250 nM (SYTO16), and 500 nM (SYTO14 and SYTO15). After 20 min incubation at RT in the darkness, 500 µl of PBS containing 2% FBS (EuroClone) was added and cells were immediately subjected to the flow cytometric analysis.

Assay conditions for SYTO green probes were determined after dose and time-course optimization studies. No sizeable differences in the efficiency of SYTO staining were detected when dye-loading was performed in PBS, PBS supplemented with 2% FBS, HEPES or complete RPMI 1640 medium or at 0.25 - 4 x 10^6 cells/staining mixture cell density. Additionally, even 5-10 min loading at 37°C was as efficient as longer incubation times (up to 60 min tested) at 37°C, and as 20 min loading at RT.

4.7.3 YO-PRO 1 / PI assay

The staining was performed as described in 4.6.2. Analysis was as previously described (Idziorek et al, 1995). Briefly, viable cells exclude both dyes (YO-PRO 1^{neg} / PI^{neg} events), early apoptotic cells characterized by initial cell membrane permeabilization are stained only with small cation YO-PRO 1 while excluding still propidium iodide (YO-PRO 1^+ / PI^{neg} events). Cells in late stages of apoptosis and primary necrotic cells are characterized by pronounced loss in cell membrane integrity, and are thus permeable to both YO-PRO 1 and PI probes (YO-PRO 1^+ / PI^+ events).

4.7.4 Phosphatidylserine exposure assay

In some experiments (data not exploited in any of the articles) Annexin V assay (APC – conjugated; Alexis Biochemicals) was used as a confirmatory technique, assessing externalization of phosphatidylserine (PS) on the outer leaflet of the plasma membrane. Staining and analysis was performed according to manufacturer instructions. Briefly after incubation with selected drugs cells were washed with Annexin V Binding Buffer (AVBB; 10 mM HEPES/NaOH pH 7.4 supplemented with 140 mM NaCl and 2.5 mM $CaCl_2$). Next cells were resuspended in 100 µl AVBB containing permeability marker propidium iodide (PI; Sigma; 5µg/ml) and 1.5 µl of Annexin V reagent was added. After 20 minutes incubation (RT in darkness) 500 µl of AVBB was added and cells were immediately subjected to the flow cytometric analysis. Viable cells were considered as Annexin V^{neg} / PI^{neg} events (containing PS

on the inner leaflet of the plasma membrane and excluding propidium iodide), early apoptotic cells were considered as Annexin V^+ / PI^{neg} events (with externalized PS while still excluding propidium iodide), whereas cells in late stages of apoptosis and primary necrotic cells were considered as Annexin V^+ / PI^+ events (with pronounced loss in cell membrane integrity).

4.7.5 Fractional DNA content analysis

Cells were exposed to HA14-1 or Brefeldin A at a dose and for the time indicated in the respective articles. Cells were then harvested, washed twice with PBS and fixed overnight with ice-cold 70% ethanol. Subsequently cells were centrifuged and incubated with RNase A (Sigma; 300 µg/ml) and propidium iodide (PI; Sigma; 16 µg/ml) for 1h at 37ºC. The cell cycle profile was determined on a FACScan flow cytometer (Becton Dickinson).

4.7.6 Caspase activation

Caspase activation was assessed supravitally by cell permeable FLICA (Fluorescently Labeled Inhibitors of Caspases) reagent as previously described (Smolewski et al, 2001; Pozarowski et al, 2003; Skommer et al, 2006).
Cells were exposed to HA14-1 or Brefeldin A at a dose and for the time indicated in the respective articles, harvested, washed and resuspended in 100µl of fresh medium, followed by staining with FLICA reagent (FAM-VAD-FMK – pan-caspase activation or FAM-DEVD-FMK – caspase 3 activation; Alexis Biochemicals) according to the manufacturer's instructions (1h at 37°C under 5% CO_2 in darkness). Next, cells were washed twice with ice-cold PBS and counterstained with plasma membrane permeability marker propidium iodide (PI; Sigma; 5µg/ml) for 5 minutes at RT in darkness. Finally 500 µl of PBS containing 2% FBS (EuroClone) was added and cells were immediately subjected to the flow cytometric analysis.

4.7.7 Mitochondrial membrane depolarization

Loss of mitochondrial membrane potential was assessed by flow cytometry using TMRM and JC9 probes (Molecular Probes) as previously described (Pritchard et al, 2001; Castedo et al, 2002). Cells were exposed to HA14-1 or Brefeldin A at a dose and depolarization for the time indicated in the respective articles, harvested, washed and resuspended in 100µl PBS containing 150 nM TMRM or 1 µg JC9 probe. After

15 min (TMRM) or 20 min (JC9) incubation at 37°C, 500 µl of PBS containing 2% FBS (EuroClone) was added and cells were immediately subjected to the flow cytometric analysis.

4.7.8 YO-PRO 1 / TMRM assay

Combined assay allowing simultaneous detection of mitochondrial membrane depolarization and varying stages of plasma membrane integrity was applied based on TMRM and YO-PRO 1 dyes (Molecular Probes).
Cells were stimulated with Brefeldin A at a dose and for the time indicated, harvested, and resuspended in 100µl of PBS containing 150 nM TMRM (Molecular Probes) to assess the depolarization of mitochondrial membrane. After 15 min loading at 37°C, cells were counter-stained with YO-PRO 1 (250 nM; Molecular Probes) for 20 minutes at RT to simultaneously detect early and late stages of plasma membrane permeability. Following staining 500 µl of PBS containing 2% FBS (EuroClone) was added and cells were immediately subjected to the flow cytometric analysis.

4.7.9 Detection of reactive oxygen species (ROS)

Dihydroethidine (DHE) staining

Intracellular generation of reactive oxygen species (ROS) was directly determined using DHE probe (Molecular Probes) as previously described (Castedo et al, 2002). Cells were stimulated with HA14-1 at a dose and for the time indicated, harvested, and resuspended in 100µl of PBS containing 2.5 µM DHE. Following staining (20 min at 37°C) 500 µl of PBS containing 2% FBS (EuroClone) was added and cells were immediately subjected to the flow cytometric analysis.

Nonyl-acridine orange (NAO) staining

Intracellular generation of reactive oxygen species (ROS) leading to peroxidation of mitochondrial cardiolipin was determined using NAO probe (Molecular Probes) as previously described (Castedo et al, 2002). Cells were stimulated with HA14-1 at a dose and for the time indicated, harvested, and resuspended in 100µl of PBS containing 100 nM NAO. After 20 min incubation at 37°C, 500 µl of PBS containing 2% FBS (EuroClone) was added and cells were immediately subjected to the flow cytometric analysis.

4.8 Multiparameter flow cytometry assays

4.8.1 SYTO16 / TMRM / 7-AAD assay

After induction of apoptosis, cells were collected, washed twice with PBS to remove phenol-red containing RPMI media, and stained in 100 µl PBS containing SYTO16 (250nM) and TMRM (150nM) for 15 min at 37ºC in darkness. Next, samples were briefly cooled on ice to the RT, and plasma membrane permeability marker, 7-AAD (Molecular Probes), was added to a final concentration of 5 µg/ml. Samples were subsequently incubated for 5 min at RT in darkness. Finally 500 µl of PBS containing 2% FBS (EuroClone) was added and cells were immediately analyzed on the flow cytometer.

4.8.2 FLICA / TMRM / 7-AAD assay

Multiparametric assay that allows simultaneous tracking of caspase activation (by FLICA), mitochondrial membrane depolarization (by TMRM) and plasma membrane permeability (by 7-AAD) was applied as previously described (Smolewski et al, 2001; Pozarowski et al, 2003). Cells were exposed to HA14-1 or Brefeldin A at a dose and for the time indicated in the respective articles, harvested, washed and resuspended in 100µl of fresh medium, followed by staining with FLICA (FAM-VAD-FMK; Alexis Biochemicals) according to the manufacturer's instructions (1h at 37°C). Next, cells were washed twice with ice-cold PBS, resuspended in PBS containing 150 nM TMRM (Molecular Probes) and incubated for 15 min at 37°C. Cells were subsequently counter-stained with 7-AAD probe (Molecular Probes; 5 µg/ml) for 3-5 minutes at RT. Finally 500 µl of PBS containing 2% FBS (EuroClone) was added and cells were immediately subjected to the flow cytometric analysis.

4.8.3 Cell cycle specificity of caspase activation

Analysis was as previously described (Smolewski et al, 2001; Pozarowski et al, 2003). Cells were exposed to HA14-1 or Brefeldin A at a dose and for the time indicated in the respective articles. FLICA reagent (FAM-VAD-FMK; Alexis Biochemicals) was added for the last hour of HA14-1 or Brefeldin A treatment. Following FLICA staining cells were washed twice with PBS and fixed for 15 min in ice-cold 1% v/v methanol-free formaldehyde. Samples were subsequently permeabilized in 70% ethanol at -20°C for at least 2 h. Following permeabilization

cells were centrifuged and stained with propidium iodide (PI; Sigma; 10 µg/ml) in the presence of RNase A (100 µg/ml) for 1 h at 37°C. Green fluorescence of FAM-VAD-FMK was analyzed using logarithmic scale, and red fluorescence of PI using linear scale on FACScan flow cytometer.

4.9 Intracellular staining for Bcl-2

Follicular lymphoma cells were fixed and permeabilized using Fix&Perm kit (Caltag Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions and incubated in the presence of 10µl FITC-conjugated anti-Bcl-2 antibodies (Caltag Laboratories) or the FITC-conjugated IgG_1 isotype-control antibody (BD Biosciences, San Diego, CA, USA). Following 20 min incubation 500 µl of PBS containing 2% FBS (EuroClone) was added and cells were immediately analyzed on FACScan analyzer (Becton Dickinson) with 15000 events acquired per sample. Bcl-2 expression was calculated as the difference in the mean fluorescence intensity (MFI) between the anti-Bcl-2 sample and the isotype control.

4.10 Evaluation of apoptotic features using fluorescent microscopy

4.10.1 Hoechst 33342

Control and HA14-1- or Brefeldin A-treated cells were rinsed with culture media and stained with Hoechst 33342 dye (Alexis Biochemicals; 1.5 µg/ml) for 20 min at RT. Following staining, cells were wet-mounted and imaged by an Olympus IX70 microscope (equipped with a cooled Apogee KX85 CCD camera) or Olympus AX70 Provis microscope (equipped with a cooled FVII digital camera; Olympus Co. Ltd., Tokyo, Japan). 100W Hg burners were used as an epifluorescent light sources. 40x and 60x air objective lenses and appropriate fluorescence mirror units (Ex/Em for Hoechst 33342 355/465 nm) were applied to collect cell images. Cell images were captured by MicroSuite™ FIVE imaging software (Olympus) running under Windows XP Professional (Microsoft Corp., Redmond, WA, USA).

4.10.2 SYTO 16 / TMRM / Hoechst 3342

HF1A3/HF4.9 control cells, or cells treated with 1 μM Dex, were rinsed with culture media and stained for 15 min with 150 nM TMRM (Molecular Probes) and 2500 nM SYTO16 (Molecular Probes) in RPMI medium at 37ºC. Subsequently cells were transferred to the RT and counter-stained with Hoechst 33342 (Alexis Biochemicals; 1.5 μg/ml) for additional 20 min. Following staining, cells were wet-mounted and imaged by an Olympus AX70 Provis microscope equipped with FVII digital camera (Olympus Co. Ltd., Tokyo, Japan). Air objective lenses (60x) and appropriate fluorescence mirror units (Ex/Em for: SYTO16 490/520 nm, TMRM 530/615 nm, Hoechst 33342 355/465 nm) were applied for obtaining respective images. Cell images were captured by MicroSuite^{TM} FIVE imaging software (Olympus) running under Windows XP Professional (Microsoft).

4.11 Confocal microscopy

For SYTO/TMRM co-localization studies, $1x10^6$ HF1A3 cells were stained with 250 nM SYTO16 and 150 nM TMRM (Molecular Probes) in PBS for 15 min at 37ºC. Following staining, cells were imaged using Nikon Eclipse inverted microscope equipped with UltraVIEW confocal scanning system (Perkin Elmer, USA). Cell images were captured and analyzed by Perkin Elmer Imaging Suite version 5.5 software running under Windows 2000 (Microsoft Corp.).

4.12 Transmission electron microscopy (TEM)

HF1A3 cells ($1x10^6$ cells/ml) were cultured with or without BFA for 24h, harvested and fixed. Sample processing for electron microscopy was carried our by the BioMater Centre at the University of Kuopio according to standard protocols. Briefly, the post-fixed sections were dehydrated in a series of ethanol and embedded in Epon resin (Ladd Research Industries, USA). The polymerized resin blocks were sectioned with a Reichert Ultracut-E ultramicrotome (Reichert-Jung, Austria). 50 nm sections were subsequently collected onto copper grids and stained with uranyl acetate. Specimens were examined under a JEOL JEM1200EX transmission electron microscope (JEOL, Tokyo, Japan) using 80 kV accelerating voltage and 14 sec exposure time. Digital micrographs were collected using BioScan 792 digital camera

(GATAN, Munich, Germany) controlled by Digital Micrograph 3.1 software (GATAN, Coronado Lane, Pleasanton, CA).

4.13 Western blotting

Control and BFA-treated cells were resuspended in the lysis buffer (Cell Signaling Technologies, Beverly, MA, USA), and proteins isolated according to manufacturer's instructions. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech; Buckinghamshire, UK). Membranes were incubated at +4ºC overnight with anti-PARP (SantaCruz Biotechnology Inc, Santa Cruz, CA, USA), anti-caspase 2 (SantaCruz Biotechnology), anti-BIP (Abcam), and anti-actin (Sigma) primary antibodies. The ER residual chaperone BIP/GRP78 was chosen as a marker of ER stress-induction by Brefeldin A, prior to electron microscopy analysis of BFA-treated follicular lymphoma cells.
Corresponding peroxidase-conjugated secondary antibodies were from Zymed (San Francisco, CA, USA). Immunodetection was performed with ECL Western blotting kit (Amersham Pharmacia Biotech).

4.14 Data analysis

4.14.1 Flow cytometry data analysis

Flow cytometry data was analyzed and presented using WinMDI ver. 2.8 software developed by Dr J. Trotter (BD Pharmingen, San Diego, CA; freely available at http://facs.scripps.edu/software.html), offline Summit v3.1 software (Dako Cytomation, Fort Collins, CO, USA) both running under Windows XP Professional operating system (Microsoft) and CellQuest ver.3.3 software (BD Biosciences, San Jose, CA, USA) running under MacOS 8.1 operating system (Apple, Cupertino, CA, USA).

4.14.2 Image analysis

Image analysis and presentation was carried out using ImageJ open source platform (developed at the National Institutes of Health, Bethesda, USA; freely

available at http://rsb.info.nih.gov/ij/) running under Windows XP Professional (Microsoft) and Perkin Elmer Imaging Suite version 5.5 software (Perkin Elmer) running under Windows 2000 (Microsoft Corp.).

4.14.3 Drug dose response analysis

Sigmoidal dose-response curves were fitted to the mean cell viability plotted against log HA14-1 or Brefeldin A dose and lethal concentration 50% (LC_{50}) values were calculated from the resulting curves using Prism 4.0 software (GraphPad Software Inc.).

4.14.4 Combination index calculations

Fractional survival was calculated as a fraction of cells killed by the individual drug or the combination in drug-treated versus untreated cells, and data were fitted to the Hill equation (Prism 4.0, GraphPad Software Inc.). Logarithmic transformation of the Hill equation (as done in the median-effect plot analysis by Chou and Talalay) wasn't performed due to reported reduced accuracy and precision of the regression-derived IC_{50} and curve shape parameters (Zhao et al, 2004). The combination index (CI) values were calculated based on the following equation:

$$CI= (D)1(Dx)1 + (D)2/(Dx)2 + (D)1(D)2/(Dx)1(Dx)2$$

where (D)1 and (D)2 are the doses of drug 1 and drug 2 that have x effect when used in combination, and (Dx)1 and (Dx)2 are the doses of drug 1 and drug 2 that have the same x effect when used alone. The CI values indicate synergism (<1), additivity (=1) or antagonism (>1). The CIs of 0.1-0.3, 0.3-0.7 and 0.7-0.85 are considered to indicate strong synergism, synergism and moderate synergism, respectively.

4.14.5 Statistical analysis

Results shown on dot plots and photographs are representatives of at least three independent experiments and SD values between experiments did not exceed ± 7%. Student's t-test was applied for comparison between groups using SPSS (Chicago, IL, USA) version 11.0 for Windows. Significance was set at $p<0.05$. Pearson correlation analysis was performed using Excel 2000 software (Microsoft).

5. Results and discussion

5.1 Small molecule Bcl-2 inhibitor HA14-1 induces apoptosis and potentiates activity of selected anti-cancer drugs in follicular lymphoma cells (articles I & II)

In many tumors the physiological capacity to remove damaged/superfluous cells is subdued, either because a pathological overexpression of prosurvival family members is present or because p53 dependent pathway is inactivated. Nevertheless a gross majority of tumors maintain components of death machinery. Thus, an attractive therapeutic approach is to overcome the intrinsic resistance to apoptosis and activate existing cell demise pathways (Spurgers et al, 2006). Due to the reported importance of the anti-apoptotic Bcl-2 family members in a multistep carcinogenesis, they have recently attracted considerable attention as forthcoming targets for selective anti-cancer regimens (Letai, 2005). Especially the family of small organic molecules (BH3-mimetics) able to bind to the hydrophobic groove of Bcl-2/Bcl-X_L and displace Bax/Bak and BH3-only proteins, may represent a future avenue for a selective anti-Bcl-2 strategy (Cory and Adams, 2005; Letai, 2005; Letai, 2006).

In this context, we have for the first time demonstrated monotherapeutic potential of a small molecule Bcl-2 antagonist, HA14-1, against follicular lymphoma cells with t(14;18) translocation and resulting Bcl-2 overexpression. HA14-1, a recently discovered cell permeant and nonpeptidic compound able to trigger dissipation of the mitochondrial membrane potential and cytochrome c release, was one of the first Bcl-2/Bcl-X_L antagonists developed by virtual library screening (Wang et al, 2000). Moreover, low concentrations of HA14-1 were reported to exert minimal toxicity towards normal human hematopoietic cells, presenting a potential therapeutic window (Lickliter et al, 2003).

5.1.1 Monotherapeutic potential of HA14-1 against follicular lymphoma cells

In agreement with previous studies on a number of hematopoietic malignancies, we showed for the first time that follicular lymphoma cells are sensitive to HA14-1-induced cell killing. HA14-1 cytotoxicity was mediated through apoptosis, as depicted by nuclear fragmentation, morphological changes, reduced SYTO 16 fluorescence

and reduction of DNA content to sub-G1 levels. In all FL cells examined, HA14-1 induced a prominent decline in cell viability, however, the HF4.9 cell line was the most sensitive to HA14-1-mediated cytotoxicity (LC_{50} of 4.5μM compared to LC_{50} of 12.6 μM for HF1A3 and LC_{50} of 8.1 μM for HF28RA cell lines), featuring at the same time the highest expression of Bcl-2 protein.

Consistent with previous reports, we showed that cytotoxicity mediated by a small molecule Bcl-2 inhibitor HA14-1 in FL cells proceeded through rapid (4h) dissipation of the $\Delta\Psi_{m,}$ elaborate generation of reactive oxygen species (ROS) and caspase activation. Although contradictory reports about HA14-1-induced caspase activation do exist in the literature, the HA14-1 mediated cell killing of FL cells was fully inhibited by means of pharmacological caspase inhibitors (Wang et al, 2000; Chen et al, 2002; An, et al, 2004). Both Z-VAD-fmk (inhibitor of broad range caspases), Z-LEHD-fmk (inhibitor of caspase 9), and Z-DEVD-fmk (inhibitor of caspase 3) completely abrogated the oligonucleosomal DNA fragmentation. Of importance, pan-caspase inhibitor z-VAD-fmk abolished both morphological changes and loss of cell membrane integrity evaluated by FSC/SSC changes and PI uptake by flow cytometry. Our results are in line with recent reports by Chauhan et al (2006) and van Delft et al (2006) reporting complete caspase dependence of the novel small molecule BH3 mimetic ABT-737 in multiple myeloma cells and murine embryonic fibroblasts, respectively. Interestingly, novel findings identify also that HA14-1 can induce both apoptosis and autophagy in L1210 murine leukemia cells and in the absence of caspase activation HA14-1-mediated cell death is shifted towards autophagic program (Kessel and Reiners, 2006).

5.1.2 Mechanisms of HA14-1-induced apoptosis

The temporal and quantitative relationship between mitochondrial membrane depolarization and caspase activation still remains ambiguous. Moreover it is still debatable whether caspase activation is restricted only to cells with mitochondrial membrane depolarization, or can occur autonomously. In this context, it has recently been proposed that the HA14-1-evoked loss of $\Delta\psi_m$ is insufficient for the initiation of full apoptotic response in the absence of caspase cascade activation (Milella et al, 2002). Following this lead, we investigated the quantitative relationship between $\Delta\Psi_m$ loss and caspase activation in HA14-1 challenged FL cells. We employed both pharmacological inhibitor studies and novel state-of-the-art multiparametric flow

cytometry assay combining simultaneous tracking of mitochondrial membrane depolarization (by TMRM probe), caspase activation (by FLICA probe) and cell membrane permeability (by 7-AAD probe), analogical to one used by Pozarowski et al (2003). Tracking kinetic events in HF4.9 cells revealed that following HA14-1 treatment caspase activation occurs solely as a consequence of mitochondrial breach. In line with others (Milella et al, 2002), the kinetic study revealed also that at longer exposition times (over 24h) mitochondrial breach slightly lessened (indicative of transient nature of $\Delta\psi_m$ loss), whereas the number of cells with activated caspases constantly increased over time. As a result, at 48h time point the majority (approx. 92%) of cells with $\Delta\psi_m$ loss featured caspase activation. Importantly, a very careful monitoring of early changes (0-4h in 15 min intervals) following HA14-1 treatment did not allow detection of population with caspase activation and preserved $\Delta\psi_m$. In agreement with our previous results pre-treatment with a pan-caspase inhibitor zVAD-fmk prevented caspase activation although did not obstruct the HA14-1-elicited $\Delta\psi_m$ loss. Additionally, hindering mitochondrial permeability transition pore (PTP) with cyclophilin D inhibitor (cyclosporin A, CsA) partially protected HF4.9 cells against HA14-1 induced $\Delta\psi_m$ collapse and caspase activation. Thus, we provided new evidence that mitochondrial apoptosis induced by a small molecule Bcl-2 inhibitor may be at least partially dependent on PTP.

In the context of molecular mechanisms underlying Bcl-2 antagonists' action, a recent report by van Delft and colleagues (2006) argues that many BH3 mimetics, including HA14-1, BH3I-1, Compound 6, Antimycin A, Chelerythrine and Gossypol, do not reflects BH3-only proteins mediated cell killing. Data provided by authors indicate that above mentioned putative BH3 mimetics potently induce cell killing in Bax/Bak DKO MEFs in both short term and long term colony formation assays. It is a very intriguing finding, since Bax -/- and Bax -/- Bak -/- MEFs have been previously reported resistant to HA14-1 cell killing (Chen et al 2002; An et al, 2004). Moreover, Bax translocation to mitochondria was reported compulsory for HA14-1-elicited cell demise (Chen et al 2002; An et al, 2004). Undoubtedly further studies are required to understand molecular basis of apoptosis induction by BH3 mimetics and to develop novel, more specific protein-protein disruptors.

5.1.3 Cell cycle specificity of HA14-1-evoked apoptosis

The knowledge on a cell cycle specificity of apoptosis induction is of clinical importance by offering hints how to design effective multidrug treatment regimens. Moreover, since cell cycle preferential killing may depend on cell type, combining drugs that target distinctive phases of the cell cycle is likely to be therapeutically beneficial. While the definite phase of the cell cycle at which HA14-1-treated cells are apoptosing has not been addressed, we employed a flow cytometric assay based on bivariate analysis of the DNA content and FLICA binding implemented by Pozarowski and colleagues (2003). In this assay cell cycle position is measured for both apoptotic and non-apoptotic cells, allowing direct verification of caspase activation in relation to the cell-cycle. It should be noted that while FLICA specificity towards individual caspases, in terms of exclusivity of their binding to individual caspases, may be questioned (Pozarowski et al 2003), application of these reagents allows for convenient dectection of caspases activation, identification of apoptotic cells and location of their position in the cell cycle. We revealed that following a low dose HA14-1 challenge follicular lymphoma cells undergo apoptosis mainly from G_1 phase, and to a lesser extent from S- and G_2/M phases. Because cell cycle specificity may substantially differ in the case of early and delayed apoptosis, we determined the effect of transient exposure of FL cells to a high HA14-1 concentration, followed by cell growth in drug-free medium. Here again the vast majority of cells underwent apoptosis mainly from G_1 and S phases of the cell cycle. Based on our results it is tempting to speculate that combining the small molecule Bcl-2 inhibitor HA14-1 with drugs arresting cells in G_1 and/or S phase, or drugs inducing apoptosis in G_2/M phase may be therapeutically beneficial. Only recently, our conclusions have been substantiated by two excellent reports from Michael Andreeff's group at MD Anderson Cancer Center in Houston (Kojima al, 2006; Konopleva et al, 2006). Similarly to us, authors illustrate that initiation of mitochondrial apoptosis in response to novel BH3 mimetic ABT-737 is constrained predominantly to cells in the G1 cell cycle phase. Based on their previous report (Konopleva et al, 2006) Kojima and colleagues deliver also convincing evidence that relative resistance of G2/M cells to the BH3 mimetic can be correlated with their higher Bcl-2 expression and Bcl-2 phosphorylation at Ser^{70}. This is of particular interest as the regulation of Bcl-2 family proteins during cell cycle remains still elusive. Illustration that the increase of Bcl-2

levels occurs with the progression from G1 to G2 phase may clarify increased sensitivity of G1 cells to both spontaneous, ABT-737- and HA14-1-induced apoptosis. Furthermore in line with our earlier conclusions, Kojima and colleagues demonstrated that small molecule Mdm2 antagonist Nutlin-3a, arresting cells in G1 and inducing apoptosis predominantly in S and G2/M phase, potently synergizes with ABT-737 in acute myeloid leukemia cells. Taken together, there is a substantial excitement that combination of novel protein-protein inhibitors will form cornerstones of novel regimens of substantial therapeutic prospective.

5.1.4 Efficacy of combinatorial treatment between the Bcl-2 antagonist HA14-1 and conventional anti-cancer chemotherapeutics

Finally, we were first to prove the applicability of a combinatorial treatment with the small molecule Bcl-2 antagonist HA14-1 and selected conventional chemotherapeutics in follicular lymphoma cells. At simultaneous exposition scenarios HA14-1 significantly increased the cytotoxicity of dexamethasone (Dex), whereas no significant enhancement of vincristine (Vin) and doxorubicin (Dox) action was observed in follicular lymphoma cells. In each cell line tested the concentrations of HA14-1, were only mildly cytotoxic when administered alone (below 15% of killing). Thus, the augmentation in lethality upon Dex/HA14-1 co-administration was beyond the effects of either of these agents alone, indicative of synergistic action. Of importance, the addition of HA14-1 induced a 2.5-6 fold increase in the early apoptotic population compared to cells exposed to Dex alone when examined by SYTO16/PI assay.

To evaluate the prospects of sequential drug administration, we subsequently challenged follicular lymphoma cells with HA14-1 for 24 hours, the medium was removed and the second cytotoxic agent (Dex, Dox or Vin) was added for another 24 hours. Cell viability was evaluated by SYTO 16/PI assay at the end of the incubation period. The results indicate that HA14-1 pretreatment sensitized FL cells to Dex and Dox and the cytotoxicity of the combinations was beyond the action of any of these agents alone. Moreover the combined effects between Vin and HA14-1 were lower than additive. The CI values, obtained from fixed molar ratio experiments, confirmed the synergy between HA14-1 and Dex (the CIs < 1 for simultaneous and sequential administration) and Dox (the CIs < 1 for sequential administration). Correspondingly to our results, the schedule dependent enhancement of paclitaxel cytotoxicity by a

novel small molecule inhibitor of Bcl-X_L A385358 has recently been reported by Shoemaker et al (2006) in small cell lung carcinoma cells.

Overall, we showed for the first time the capacity of the small-molecule Bcl-2 antagonist HA14-1 to enhance Dex and Dox-induced cytotoxicity and apoptosis in follicular lymphoma cell lines. Concentrations of the HA14-1 used in our experiments were substantially below the previously reported *in vitro* toxic level for normal hematopoietic cells and these findings suggest prospective clinical effectiveness of BH3 mimetics. Results of our study indicated for the first time not only the single agent efficacy of BH3 mimetic in follicular lymphoma cells but also suggested that combinatorial treatment with BH3 mimetics may be an efficient strategy to render cytotoxic agents more efficient at lower systemic doses. In this context, oblimersen sodium (G3139) and (-)gossypol (a natural small molecule inhibitor of Bcl-2/Bcl-X_L) have been shown to enhance response to VAD (vincristine, adriamycin and dexamethasone) regimen in multiple myeloma patients, and to CHOP (cyclophosphamide-doxorubicin-vincristine-prednison) regimen in diffuse large cell lymphoma cells, respectively (van de Donk et al, 2004; Mohammad et al, 2005). Of particular interest, ours and others findings have been recently substantiated by a report demonstrating synergistic action between Dex and a novel synthetic BH3 mimetic ABT-737 in multiple myeloma cells (Chauhan et al, 2006). Furthermore, very recent reports by Oltersdorf et al (2005), Konopleva et al (2006) and van Delft et al (2006) delivered unprecedented data to support the notion of combinatorial treatment with the small molecule Bcl-2 antagonist ABT-737 and selected conventional chemotherapeutics (doxorubicine, cisplatine, paclitaxel, Ara-C, etoposide) in a variety of tumor models.

5.2 ER-Golgi network as a novel anti-cancer target in follicular lymphoma (article IV)

A growing number of reports recognize endoplasmic reticulum (ER) and Golgi apparatus as key players in the sensing and execution of cell demise signals. As the interest in the role of ER and Golgi during induction/execution of apoptosis has been gaining momentum, they also attract growing attention in the development of novel targets for selective anti-cancer therapies (Ferri and Kroemer, 2001; Linder and

Shoshan, 2005; Carew et al, 2006). In this context, it has been recently demonstrated that malignant B-CLL blasts feature enlarged mitochondrial and ER compartments as compared to their normal counterparts (Carew et al, 2006). Postulated theory links elaborate ER-Golgi system to the endurance of malignant clones and suggests unique anti-cancer target with reasonable therapeutic window (Carew et al, 2006).

Hence, we have for the first time demonstrated monotherapeutic potential of ER-Golgi transport disrupting agent, Brefeldin A (BFA), against follicular lymphoma cells bearing t(14;18) translocation. In agreement with previous studies on a number of hematopoietic malignancies and solid tumors, we provided for the first time evidence on nanomolar efficacy of Brefeldin A against follicular lymphoma cells. In the long-term (5-day) growth experiments we revealed that the administration of low doses of BFA (25 ng/ml) completely inhibits growth of HF4.9 and HF28RA cells. Somewhat higher BFA doses (75 ng/ml) were required to achieve the same effect in HF1A3 cells. Moreover, short-term studies (24h) provided strong evidence that cell proliferation was inhibited in a dose-dependent manner and, depending on the cell line, almost complete cessation of ^{3}H-thymdine incorporation was observed at 50-75 ng/ml of BFA. By implementing YO-PRO1/PI viability assay, we also revealed that both diminished cell proliferation and enhanced cell death appeared to associate in the long-term growth cessation triggered by BFA.

Brefeldin A-mediated cell killing proceeded through apoptosis, as represented by a plethora of markers including: chromatin condensation and nuclear fragmentation, cell shrinkage, reduced SYTO 16 fluorescence, selective uptake of cationic probe YO-PRO1, Annexin V binding and reduction of DNA content to sub-G1 levels. Moreover, the distinctive apoptotic morphology was accompanied by caspase activation, as determined by immunoblot analysis of PARP (poly-ADP-ribose polymerase) cleavage and by FLICA (herein FAM-VAD-FMK) staining.

Interestingly, contradictory reports about BFA-induced caspase activation exist in the literature. Herein pre-treatment with a pan-caspase inhibitor zVAD-fmk at 50μM, rescued BFA-treated FL cells only partially. It is of special interest as previous results employing FLICA assay depicted clearly that the entire apoptosing population in BFA-treated cells featured caspase activation. We have always obtained similar results whichever FLICA reagents were employed (FAM-VAD-FMK or FAM-DEVD-FMK). Because apoptotic cascade can be initiated from Golgi apparatus

through caspase 2 which is approximately 55 times less sensitive to inhibition by zVAD-fmk (compared to caspase 3/7), we examined caspase 2 processing by Western blotting in BFA stimulated FL cells (Garcia-Calvo et al, 1998; Hicks and Machamer, 2005). Indeed, noticeable reduction in levels of procaspase-2 was detected upon BFA stimulation in all cell lines tested which is in line with several previous reports showing the involvement of caspase 2 in BFA-mediated cell demise (Carew et al, 2006; Cheung et al, 2006). Moreover, any significant protection against BFA-triggered cell death was conferred by neither cathepsin nor calpain inhibitors (MDL28170, PD150606, CA-074-Me, zFA-fmk, and ALLN) applied in the present study. Protein synthesis inhibitor cycloheximide (CHX) was able to significantly restore viability of HF1A3 and HF4.9 cells (Fig. 10), whereas ROS scavengers, ascorbic acid and N-acetyl cysteine, exerted no protective effects (Fig. 11). Although appealing, data from CHX studies should be interpreted with considerable caution. It is very plausible that reduced protein load to the ER and, thus, reduced ER stress may reflect enhanced viability in BFA stimulated cells.

Fig. 10 Protein synthesis inhibitor cycloheximide (CHX) confers partial protection against BFA-induced cell death.
FL cells were pre-incubated for 1 hour with 100 ng/ml CHX. Subsequently, BFA was added to a final concentration of 100 ng/ml and cells were incubated for the 24 hours. As a control FL cells were stimulated with 100 ng/ml CHX or 100 ng/ml BFA alone. Cell viability was determined by SYTO16/PI and YO-PRO 1/PI staining, as described under Materials and Methods. The results represent mean ± SD of at least three independent experiments.

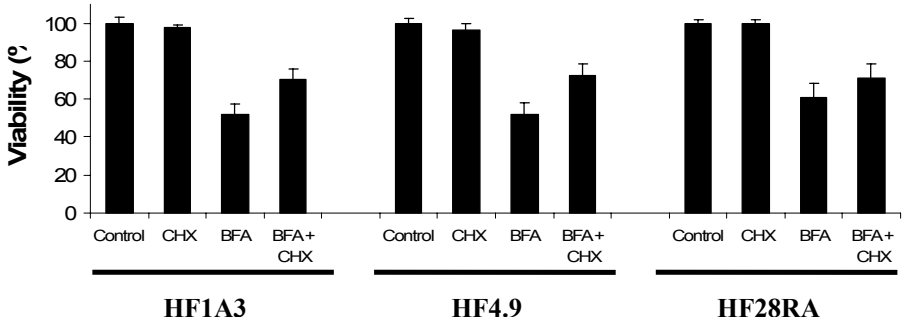
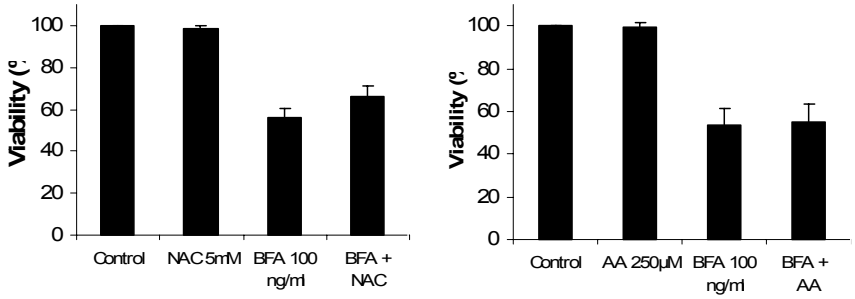


Fig. 11 Analysis of ROS dependence in Brefeldin A (BFA)-induced cell death. A) HF1A3 cells were pre-incubated for 2 hour with 5 mM N-acetylcysteine (NAC). Subsequently, BFA was added to a final concentration of 100 ng/ml and cells were incubated for the 24 hours. As a control FL cells were stimulated with 5 mM NAC or 100 ng/ml BFA alone. Cell viability was determined by SYTO16/PI and YO-PRO 1/PI staining, as described under Materials and Methods. The results represent mean ± SD of at least three independent experiments. B) HF1A3 cells were pre-incubated for 2 hour with 250 µM ascorbic acid (AA). Subsequently, BFA was added to a final concentration of 100 ng/ml and cells were incubated for the 24 hours. As a control FL cells were stimulated with 250 µM AA or 100 ng/ml BFA alone. Cell viability was determined by SYTO16/PI and YO-PRO 1/PI staining, as described under Materials and Methods. The results represent mean ± SD of at least three independent experiments.

A) B)



Quantitative relationship between $\Delta\Psi_m$ loss and caspase activation in BFA challenged FL cells revealed that mitochondrial breach proceeded slowly during the 24 hours of BFA treatment, and was accompanied by increased FLICA binding, indicative of caspase activation. Similarly to HA14-1 studies (refer to chapter 5.1) we were unable to detect cell population with activated caspases and preserved mitochondrial membrane potential, suggesting that mitochondrion stands at the nexus of apoptotic program also in this model. Almost all cells with $\Delta\psi_m$ collapse appeared to have activated caspases and upon BFA removal these cells did not recover but instead progressively acquired late apoptotic/necrotic phenotype (characterized by cell membrane permeability to 7-AAD). Our data support the hypothesis that MMP is a rate-limiting step during BFA-induced cell killing and are in line with earlier report showing that two distinct MMP inhibitors (Bcl-X_L and vMIA) can significantly

protect against BFA-, tunicamycin- and thapsigargin-induced apoptosis (Boya et al, 2002).

While the cell cycle dependence of apoptosis initiation by ER and/or Golgi stressors has not been investigated, we employed a flow cytometric assay based on bivariate analysis of the DNA content and FLICA binding (refer to chapter 5.2 and Pozarowski et al, 2003) to track caspase activation in relation to the cell-cycle position. Cell cycle specificity studies revealed that BFA induced caspase activation from all cell cycle phases, but with clear predominance of cells at G1 phase. To the best of our knowledge this was the first study to address this issue with respect to BFA-induced cell death.

Since a large number of reports have demonstrated that elevated Bcl-2/Bcl-X_L/Mcl-1 expression is associated with a poor clinical response, a combinatorial strategies aimed at diverse apoptotic elements may offer substantial therapeutic promise. Brefeldin A has been previously suggested as an investigational anticancer agent targeting secretory pathway, however its potential interactions with other apoptosis-inducing compounds remain surprisingly unexplored (Sausville et al, 1996; Shao et al 1996; Chapman et al, 1999; Carew et al, 2006). Merely one study by Shao and colleagues (1996) reported synergistic induction of apoptosis upon combinatorial treatment of human promyelocytic leukemia cells with BFA and staurosporine. Hence, we investigated the potential therapeutic utility of combined treatment between BFA vs. a small molecule Bcl-2 inhibitor (HA14-1) and BFA vs. a model trigger of death-receptor pathway (CD95 cross-linking mAb). As discussed in the chapter 5.2, we have previously demonstrated the potential of BH3 mimetic HA14-1 to induce apoptosis in a single-agent and combinatorial treatment scenarios. Strikingly, upon co-treatment of FL cells with HA14-1 and BFA we observed an enhanced cell killing, whereas the co-stimulation anti-CD95 mAb increased the efficacy of Brefeldin A to a much lesser degree. Together, these data clearly point out that combinatorial targeting of diverse cell death pathways may improve anticancer action of ER/Golgi stressors. It would be extremely interesting to test this premise further in primary patient-derived cells and *in vivo* models.

In closing, the secretory pathway attracts slowly mounting interests as a prospective anti-cancer target. Our results support the premise that malignant B-lineage cells, engaged in intense secretory function, are highly vulnerable to the interruption of ER-Golgi homeostasis. Recent studies by Tinhoffer et al (2002) and Anether et al (2003) have delivered novel insights into elimination of fludarabine refractory B-CLL primary cells and T-acute lymphoblastic leukemia cells by Tetrocarcin A, an another naturally occurring antibiotic that initiates apoptosis through ER-stress. Of particular interest, N-linked glycosylation inhibitor, tunicamycin, was recently reported to sensitize human prostate cancer cells to TNF-related apoptosis-inducing ligand (TRAIL) (Shiraishi et al, 2005). Taken together it is tempting to contemplate that ER stressors may represent a completely novel avenue in the development of future anti-cancer drugs.

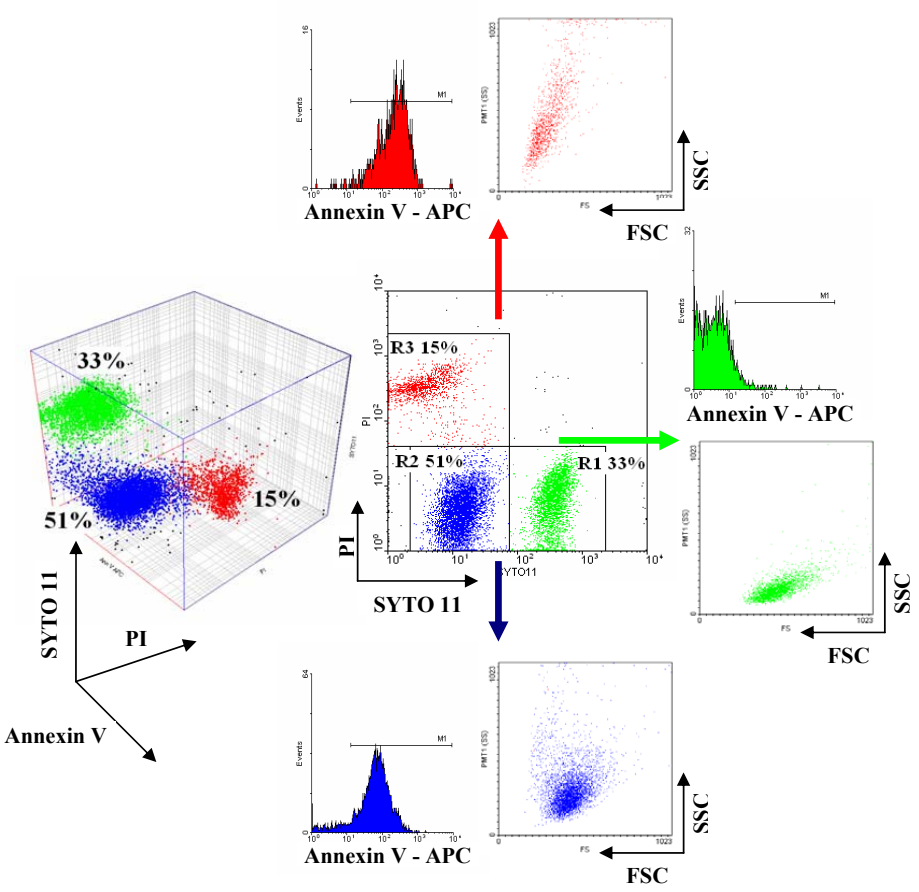
5.3 Towards an understanding of apoptosis detection by cell permeable SYTO probes (article III)

Cell permeable SYTO dyes are gaining escalating interest as sensitive, reliable and easy to use markers of apoptotic cell demise (Frey, 1995; Poot et al 1997; van der Pol et al, 2003; Sparrow and Tippett, 2005). Noticeably, the observable fact of differential SYTO staining of apoptotic cells compared to normal cells is still not fully elucidated. Moreover as a rising number of researchers apply SYTO dyes for assessment of apoptosis by flow cytometry and fluorescence microscopy, there is definitively a need for deciphering the phenomena underlying staining characteristics of those unique probes (Schuurhuis et al, 2001; van Zandvoort et al, 2002; Sparrow and Tippett, 2005).

Recent reports have shown comparable or higher sensitivity of SYTO probes as compared to Annexin V based assays (Schuurhuis et al, 2001; Sparrow and Tippett, 2005). Herein, we have reconfirmed those results by applying for the first time a triplicate staining with Annexin V-APC/SYTO 11/PI probes (Fig 12).

In the present survey we also showed that SYTO dyes from the green family (SYTO11-16) have overlapping staining characteristics, supplementing the findings reported by Poot and colleagues (Poot et al, 1998).

Fig. 12 Similarities between SYTO 11 and Annexin V staining patterns upon induction of apoptosis. HF4.9 cells were exposed to 1 μM Dex for 24 hours, followed by the triplicate staining with SYTO11, Annexin V (APC conjugated) and propidium iodide (PI) probes. The bivariate distribution of SYTO 11 vs. PI was derived from the 3D plot. Histograms and FSC/SSC plots were derived from respective gates (R1, R2, R3) drawn on SYTO 11/PI bivariate plot. Green events (R1) - live cells, Blue events (R2) – apoptotic cells, Red events (R3) – late apoptotic/necrotic cells (PI-positive). Note that cells with loss of SYTO 11 fluorescence to dim values predominantly stain with Annexin V. Three independent experiments yielded comparable results. Similar results were obtained in two other cell lines, HF1A3 and HF28RA (not depicted).



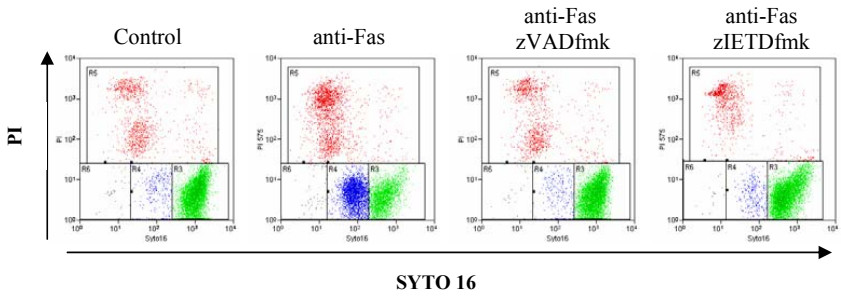
We demonstrated that SYTO11, 13, 14, and 16 probes allowed a coherent discrimination of viable ($SYTO^{high}/PI^{-}$), apoptotic ($SYTO^{dim}/PI^{-}$) and late

apoptotic/necrotic ($SYTO^{low}/PI^{+}$) subpopulations, whereas SYTO12 and 15 could evidently distinguish the same subpopulations only on FSC/SYTO bivariate plots. The discrimination of viable, apoptotic and late apoptotic/necrotic cells by SYTO probes was confirmed after back-gating every subpopulation onto FSC/SSC and FSC/PI bivariate plots. Interestingly, the number of $SYTO^{dim}$ cells after staining with any of SYTO11, 13, 14 and 16 dyes correlated well ($r^2 \geq 0.98$; Pearson and Lee linear correlation test). The lack of the decrease in SYTO15 fluorescence to dim values in response to apoptotic trigger was in general accordance with study by Poot and colleagues (Poot et al, 1998). To the best of our knowledge, however, this was the first report showing the clear discrimination of viable, apoptotic and late apoptotic/necrotic populations based on the bivariate FSC *vs.* SYTO12/15 dot plots.

In the present report, applying both the state-of-the-art multiparametric flow cytometry and multicolor cell imaging, we identified also for the first time different SYTO staining characteristics upon apoptotic and oncotic stimuli. It has been showed previously that SYTO probes are specific markers of caspase dependent apoptotic cell demise (Fig. 13) and loss of SYTO16 fluorescence can be considered as a truly apoptotic feature (Poot et al, 1997; van der Pol et al, 2003; Sparrow and Tippett, 2005). Interestingly there are currently no reports describing behavior of SYTO dyes under oncotic/necrotic conditions or during autophagic or caspase independent cell death modes. Herein, we showed that rapid loss of the SYTO16 fluorescence to low values is noticeable after challenging FL cells with diverse oncotic stimuli. We observed that exposition of cells to relatively harsh oncotic stimuli (1% NaN_3 – 4 hours, 56°C – 5 min) resulted in manifestation of predominant $SYTO^{low}/PI^{+}$ phenotype, representative of cells with a compromised plasma membrane. Apparent enlargement in the number of $SYTO^{high}/PI^{-}$ cells was also observable after stimulating FL cells with sodium azide or exposing them to +46ºC.

Fig. 13 Analysis of caspase dependence in SYTO16 staining profiles after induction of apoptosis in FL cells.
HF1A3 cells were pre-incubated for 1 hour with 20 µM pan-caspase inhibitor (zVADfmk) or 20 µM caspase 8 inhibitor (zIETDfmk). Subsequently, agonistic anti-Fas mAb (clone CH11) was added to a final concentration of 10 ng/ml and cells were incubated for the 24 hours, followed by staining with SYTO16 and PI probes as described in Materials and Methods section. As a control FL cells were treated with

10 ng/ml anti-Fas mAb or 20 μM zVADfmk/ zIETDfmk alone. Cell debris showing extremely low FSC/SSC values were excluded electronically from each plot. Results are representative of four independent experiments.

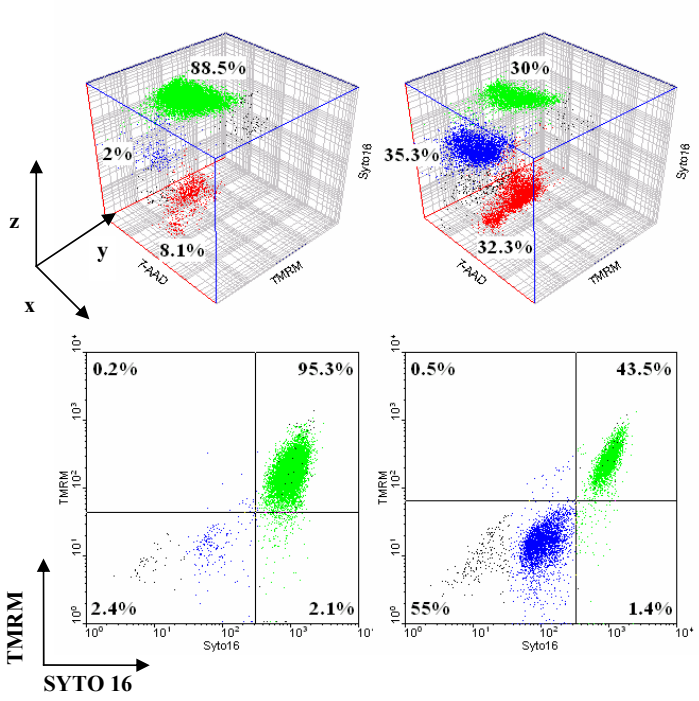


Finally, prompt by recent reports, we also tracked similarities and divergences between SYTO16 and TMRM (tertramethylrhodamine methyl ester; $\Delta\Psi_{m,}$ sensitive probe) (Poot et al, 1997; Sparrow and Tippett, 2005). Although simultaneous loss of both dyes has been previously observed by some authors and this has even led to the proposal of SYTO staining dependence on $\Delta\Psi_m$ we hypothesized that differences between staining properties of SYTO16 and TMRM can be unveiled upon shorter treatment settings (Broxterman et al, 1997; Sparrow and Tippett, 2005). When stimulated with conventional inducers of apoptosis (Dex, CHX, agonistic anti-Fas mAb) the loss of SYTO16 and TMRM fluorescence occurred concomitantly (Fig. 14). Interestingly, even during short-term kinetic experiments with those agents, we continuously noticed only parallel loss in fluorescence of both probes. No intermediate events ($SYTO^{dim}/TMRM^{high}$ and/or $SYTO^{high}/TMRM^{low}$) were observed between apoptotic and normal cells.

To validate our findings from multiparameter flow cytometry, we have also performed fluorescence microscopy after tri-color staining with SYTO16 / TMRM / Hoechst 33342 probes, as described in Materials and Methods. Upon treatment with Dexamethasone or agonistic anti-Fas monoclonal antibody, dying cells displayed loss of tertramethylrhodamine methyl ester staining (considered as a marker of $\Delta\psi_m$ loss) and reduction of SYTO fluorescence (reflecting "dim" subpopulation previously distinguished by flow cytometry). All $SYTO^{dim}/TMRM^{-}$ cells exhibited enhancement of Hoechst 33342 fluorescence with apparent apoptotic features: fragmentation of nucleus and cell shrinkage.

Fig. 14 Resemblance between SYTO16 vs. TMRM staining profiles after induction of apoptosis in FL cells.

HF1A3 cells were left untreated (control) or stimulated with Dexamethasone (Dex; 1 µM, 24h), followed by triplicate staining with SYTO16, TMR and /7-AAD probes as described in Materials and Methods section. 3D plots and bivariate SYTO16 vs TMRM plots (after electronic exclusion of debris and 7-AAD$^+$ events) indicate concomitant and progressive loss of SYTO and TMRM fluorescence. x, y and z axis on the 3D plots represent 7-AAD, TMRM and SYTO16 fluorescence respectively. Note the excellent correlation between number of SYTO16^{low}/7-AAD$^-$ and TMRM^{low}/7-AAD$^-$ events. Cell debris showing extremely low FSC/SSC values were excluded electronically from each plot. Results are representative of four independent experiments. Similar results were obtained with HF4.9 and HF28RA cell lines (not depicted).



Supplementary confocal microscopy analysis was performed to resolve the extent of SYTO16/TMRM co-localization. We observed that in fact SYTO16 co-localized with TMRM only partially and quantitative analysis indicated that 21-45% of SYTO16

stains independently of TMRM. Guided by the results, we therefore assumed that at least under some circumstances it should be possible to monitor different staining profiles between SYTO16 and $\Delta\Psi_{m,}$ sensitive probe TMRM.

In this context we demonstrated for the first time that stimulation of FL cells with mitochondrial uncoupler FCCP and a small-molecule Bcl-2 inhibitor, HA14-1, induce distinct staining profiles with the decrease in TMRM fluorescence preceding the loss of SYTO16 fluorescence. The initial loss of TMRM fluorescence upon HA14-1 treatment appeared to precede the loss of SYTO16 fluorescence, the latter trailing to dim values over longer exposure time. Importantly, FCCP and HA14-1-mediated reduction of SYTO16 fluorescence was blocked by pharmacological inhibition of caspases (with a pan-caspase inhibitor, z-VAD-fmk). Our data reveal, thus, that loss of SYTO16 appears to be a caspase-dependent event and loss of SYTO fluorescence to dim values is not a mere indicator of $\Delta\psi_m$ dissipation, postulated by some authors (Broxterman et al, 1997; Sparrow and Tippett, 2005). Moreover, we provided strong evidence that commonly observed similarities between SYTO and TMRM may stem from the fast kinetics of apoptotic events once cell death program is already initiated.

6. Concluding remarks

A. Small molecule Bcl-2 inhibitor HA14-1 induces apoptosis and synergizes with selected anti-cancer drugs in follicular lymphoma cells (articles I & II)

We have for the first time shown that follicular lymphoma cells are sensitive to HA14-1-induced cytotoxicity mediated through the intrinsic apoptotic pathway. The cell killing induced by a small molecule Bcl-2 inhibitor HA14-1 proceeded through rapid dissipation of the $\Delta\Psi_{m,}$ generation of reactive oxygen species and caspase-dependent apoptosis. Based on results obtained from pharmacological inhibitor studies and novel state-of-the-art multiparametric flow cytometry assays we conclude that: 1) Upon binding of HA14-1 into the BH-3 binding pocket of Bcl-2 (and possibly other Bcl-2 related proteins) caspase activation occurs only as a consequence of mitochondrial disruption; 2) HA14-1-evoked apoptosis appears to be at least partially PT-dependent, providing additional evidence to a widely discussed controversy over generality of PT as a primary mechanism for mitochondrial outer membrane permeabilization and apoptosis.

Moreover, we also pioneered in addressing the cell cycle specificity of HA14-1 action using multivariate flow cytometry approaches. As the vast majority of cells underwent apoptosis mainly from G_1 and S phases of the cell cycle, it is tempting to speculate that combining the small molecule Bcl-2 inhibitor HA14-1 with drugs arresting cells in G_1 and/or S phase, or drugs inducing apoptosis in G_2/M phase may be therapeutically beneficial.

Finally, we showed for the first time the ability of the small-molecule Bcl-2 antagonist HA14-1 to enhance susceptibility towards dexamethasone and doxorubicin-induced cytotoxicity (in schedule dependent and independent manner, respectively) and apoptosis in follicular lymphoma cell lines.

Based on our results and mushrooming reports it is tempting to speculate that synthetic inhibitors of antiapoptotic Bcl-2-like proteins are among the most promising experimental anti-cancer therapeutics of the XXI century. Further studies are unquestionably necessary to develop more potent and selective inhibitors of Bcl-2 and related proteins, and gain further insights into the “devil dance” played by the members of the Bcl-2 family.

B. Engaging secretory pathway by Brefeldin A as a novel mean to combat follicular lymphoma (article IV)

We have for the first time shown that follicular lymphoma cells are sensitive to Brefeldin A-induced cytotoxicity associated with the mitochondrial breach and activation of caspase 2. We noted also the lack of autophagic morphology, upon BFA stimulation, reportedly associated with ER stress in some other models.

In conceptual agreement with other reports, we supported here the nanomolar efficacy of Brefeldin A against follicular lymphoma cells with survival driving Bcl-2 overexpression. Our results support the premise that malignant B-lineage cells, engaged in intense secretory function, are highly vulnerable to the interruption of ER-Golgi homeostasis even if protected by pathological overexpression of anti-apoptotic proteins.

To the best of our knowledge we were also first to address the cell cycle specificity of BFA in malignant B cells. Utilizing a multiparameter flow cytometry approach we demonstrated that the immense majority of cells underwent apoptosis from G_1 phase of the cell cycle.

Finally, pertinent to the treatment of B-cell malignancies we report here for the first time the effects of an ER-Golgi stressor, Brefeldin A (BFA), alone and in combination with a small molecule Bcl-2 inhibitor HA14-1 in the human FL cell lines bearing t(14;18) translocation. Of importance for future anti-cancer regimens, small molecule Bcl-2 antagonist, HA14-1 significantly enhanced BFA-mediated cytotoxicity and apoptosis, revealing previously unexplored means to augment ER-stress mediated cell killing.

C. Mechanisms underlying SYTO staining properties in different cell death contexts (article III)

In the survey, we have for the first time presented diverse SYTO 16 staining characteristics upon apoptotic and oncotic stimuli. Moreover by comparing SYTO 16 with a $\Delta\Psi_{m,}$ sensitive probe TMRM we were first to report a drastically distinct behavior of those dyes under very short exposure of follicular lymphoma cell to a mitochondrial uncoupler FCCP and a small-molecule Bcl-2 inhibitor, HA14-1. Several important conclusions can be drawn based on our findings:

- a sole loss of $\Delta\psi_m$ apparently does not lead to a decrease in SYTO16 fluorescence, which results instead from caspase-dependent changes occurring downstream of mitochondrion during apoptosis
- commonly detected resemblance between SYTO16 and TMRM staining profiles may stem from the fact that many apoptotic stimuli induce mitochondrial rupture and engage downstream apoptotic targets within relatively short time
- amplification of protease cascade in the later stages of apoptotic cell demise leads to an even more pronounced loss of SYTO fluorescence (represented by $SYTO^{low}$ values)
- it can not be excluded that caspase-dependent alterations in mitochondria structure and/or function may contribute to the loss of SYTO fluorescence during apoptosis.

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